Pseudomonas aeruginosa transcriptome analysis in a cystic fibrosis lung model reveals metabolic changes accompanying biofilm maturation and increased antibiotic tolerance over time.

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Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* forms biofilm infections in the lungs of people with the genetic condition cystic fibrosis (CF) that can persist for decades. There are numerous *P. aeruginosa* lifestyle changes associated with chronic biofilm infection cued by the CF lung environment. These include a loss of virulence, metabolic changes, and increased antimicrobial tolerance. We have investigated *P. aeruginosa* PA14 biofilm infection over 7 d in an *ex vivo* pig lung (EVPL) model for CF, previously shown to facilitate formation of a clinically-relevant *P. aeruginosa* biofilm structure with gene expression comparable to human infection. We extracted and sequenced *P. aeruginosa* RNA from EVPL-associated biofilms 24 h, 48 h, and 7 d after infection, and compared gene expression between sequential time points. We also investigated tolerance to polymyxins across these three time points. Our results indicate that the EVPL model can maintain a *P. aeruginosa* biofilm population, which exhibits increased antibiotic tolerance, for at least 7 d. Differential expression of antimicrobial resistance-associated genes was not observed, however there was significant upregulation of sulfur metabolism and phenazine biosynthesis-related genes, as well as maintenance of a structured biofilm. These findings provide further insight into the incidence of increased *P. aeruginosa* antibiotic tolerance during infection of the CF lung, and the gene expression changes that arise as chronic infection establishes.

Introduction

45 *Pseudomonas aeruginosa* chronic biofilm infections of the cystic fibrosis (CF) lung are
46 characterised by physiological adaptations to the environment over years of infection,
47 leading to reduced phenotypic diversity and loss of virulence in late stage infections [1].
48 Specific lifestyle changes have been shown to improve *P. aeruginosa* persistence, including
49 loss of swimming motility, impaired pyoverdine production, and increased adherence [2]. *P.*
50 *aeruginosa* adaptation is driven by the selection pressures of the unique infection
51 environment, including osmotic, nitrosative, and oxidative stress, as well as interactions
52 with the microbiome and co-infecting pathogens, and frequent antibiotic exposure [3]. *P.*
53 *aeruginosa* chronic infections in the CF lung are almost impossible to eradicate once formed
54 due to the high level of antibiotic resistance exhibited, contributed to by the formation of a
55 biofilm matrix that protects the bacterial cells from not only the host immune response, but
56 also antibiotics [4].
57
58 *P. aeruginosa* anaerobic respiration during chronic infection has also been associated with
59 increased antibiotic tolerance [5]. There is a build-up of thick mucus in the CF lung that is
60 not effectively cleared, which results in mucus plugs that block the airways. Alongside the
61 oxygen reduction caused by an abundance of active host immune cells [6], this creates an
62 oxygen gradient with regions of low oxygen availability (hypoxic regions) or no oxygen
63 (anoxic regions) [7, 8]. *P. aeruginosa* has been found to occupy a hypoxic niche in the CF
64 lung, and the oxygen gradient is then further steepened by cell proliferation [9]. Although
65 there has been research to show that *P. aeruginosa* is able to use an oxygen supply in the
66 lumen to respire via microaerobic respiration at the surface of the mucus layer [10],
67 anaerobic respiration is believed to be the predominant mechanism of respiration as growth
68 is mainly in hypoxic regions [5].

69

70 The mechanisms of respiration, driven by the CF lung environment, also have an impact on
71 the changes in *P. aeruginosa* growth and physiology over time. For example, sulfur
72 metabolism has been linked to *P. aeruginosa* growth in anoxic conditions. Traditionally
73 sulfur starvation has been considered the cause of sulfur metabolism upregulation, however
74 a study of *P. aeruginosa* PA14 growth in synthetic cystic fibrosis sputum media (SCFM)
75 found that upregulation of sulfur metabolism in PA14 anaerobic biofilms was induced by the
76 lack of oxygen [11]. There is actually an increase in sulfation of the airway mucins in CF
77 compared with a non-CF lung [12, 13], in part caused by inflammation [14], that chronically
78 adapted *P. aeruginosa* can use. It has also been shown that there is enough inorganic sulfate
79 in CF sputum to facilitate growth even without the use of mucin as a source [15], thus sulfur
80 is in abundance in this environment.

81

82 Quorum sensing (QS) largely controls the phenotypic changes observed in the *P. aeruginosa*
83 biofilm population during infection. It is involved in the development of a biofilm with
84 mature architecture and is estimated to influence expression of approximately 10% of all *P.*
85 *aeruginosa* genes [16]. Expression of QS-related genes declines over time in CF, linked to the
86 reduced virulence factor production observed in chronic infection [17]. However, QS-
87 associated gene expression in *in vitro* models has been shown to be distinct from CF sputum
88 [18]. Our previous RNA-sequencing analysis of *P. aeruginosa* in an *ex vivo* pig lung (EVPL)
89 model of CF infection demonstrated that this growth environment may more accurately
90 capture *in vivo* QS regulated gene expression than *in vitro* growth in CF sputum-mimicking
91 medium [19]. The model uses EVPL bronchiolar tissue surrounded by synthetic cystic fibrosis
92 sputum media (SCFM) to recapitulate the CF lung environment [20]. We now build upon our

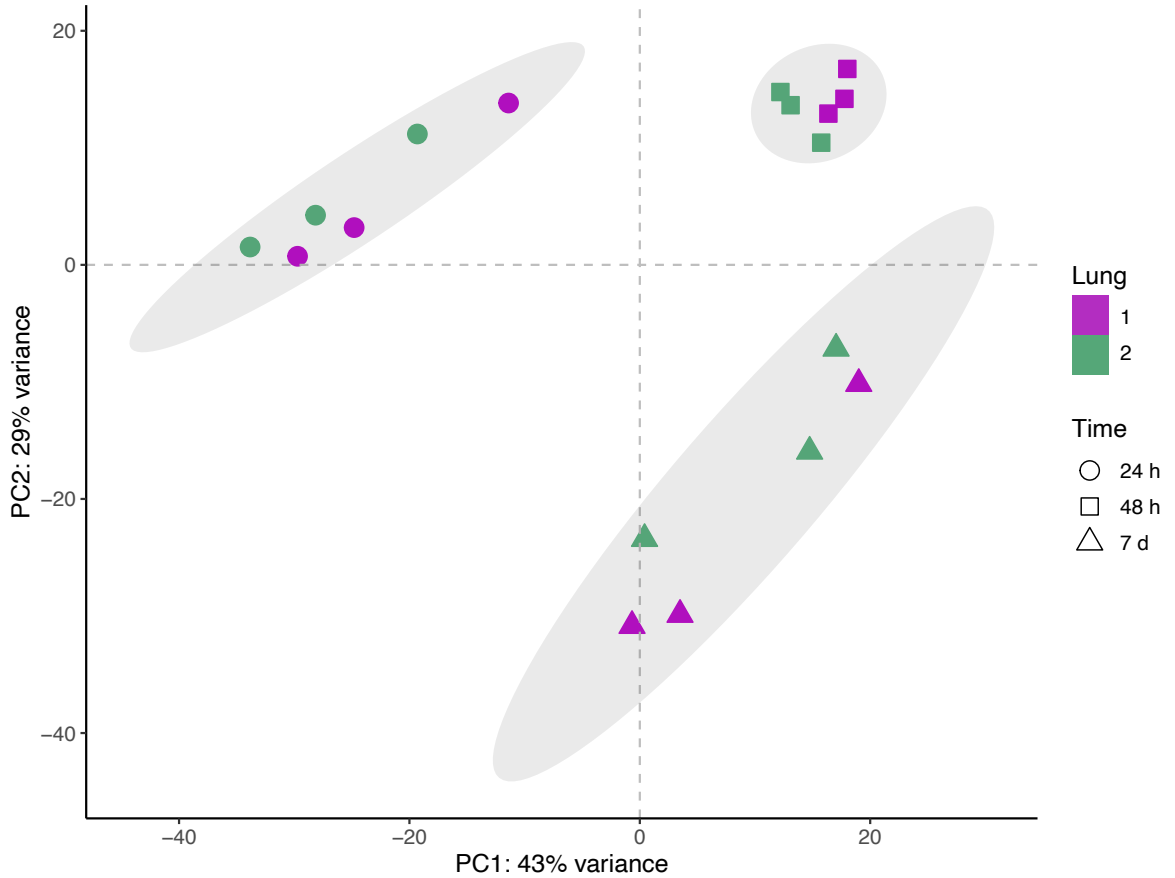
93 previously published work to investigate *P. aeruginosa* PA14 gene expression in the EVPL
94 model biofilm over 7 days, to determine the changes in expression that drive the formation
95 of a chronic biofilm infection in the CF lung.
96
97 Extended analysis of an RNA-sequencing data set, which we partially explored in our
98 previous study [19], showed that there are distinct *P. aeruginosa* transcriptomes at 24 h, 48
99 h, and 7 d in the EVPL-associated biofilm. Through differential expression analysis and Kyoto
100 encyclopedia of genes and genomes (KEGG) pathway enrichment analysis, we identified a
101 number of genes and pathways that significantly change in expression over 7 d. We also
102 investigated *P. aeruginosa* PA14 tolerance to two clinically-relevant antibiotics, colistin and
103 polymyxin B, and found that tolerance increases over time. This is not accompanied by
104 upregulation of specific antimicrobial resistance (AMR) genes but by other factors, including
105 upregulation of sulfur metabolism and the formation and maintenance of a biofilm. Hence,
106 these results provide new insight into the gene expression changes that occur as *P.*
107 *aeruginosa* antibiotic tolerance increases in the CF lung.
108

109 **Results**

110
111 *P. aeruginosa* PA14 was grown as a biofilm on the surface of EVPL bronchiolar tissue for 24
112 h, 48 h, and 7 d. The biofilm was recovered from three tissue pieces, from each of two
113 independent pig lungs, per time point. *P. aeruginosa* RNA was then extracted, and RNA
114 sequencing was performed. The colony forming units (CFU) lung^{-1} were reported in our
115 previous study, in which we compared PA14 gene expression in the EVPL model with SCFM

116 *in vitro* growth at 24 h and 48 h [19]. In the present study, we carried out differential
117 expression analysis to determine which *P. aeruginosa* PA14 genes that were significantly
118 differentially expressed ($P < 0.05$, $\log_2$ fold change ($\log_2$FC) ≥ |1.5|) over 24 h, 48 h, and 7 d
119 in the EVPL-associated biofilm. Each contrast was between two time points, and the earliest
120 time point was used as the baseline for comparison. For example, the contrast between 24
121 h and 48 h determined the $\log_2$FC in expression of genes at 48 h compared with 24 h,
122 showing the change in expression from 24 h to 48 h.
123
124 ***P. aeruginosa* PA14 had a distinct transcriptome in the EVPL biofilm at 24 h, 48 h, and 7 d,**
125 **with the majority of genes significantly overexpressed over time.**
126
127 Principal component analysis (PCA) of PA14 gene expression at each time point identified a
128 distinct transcriptome dependent on the time *P. aeruginosa* was grown on the EVPL tissue
129 for; samples from the same time point clustered together (Figure 1). The first two principal
130 components captured most of the variance between samples; principal component three
131 only accounted for 6% of the variance and its inclusion did not change the pattern observed
132 (Figure S1). There was also no clear difference in samples from different lungs, indicating
133 that these were true differences between time points and were not caused by lung
134 variation. *P. aeruginosa* grown for 24 h appeared to be the most distinct in principal
135 component 1, which accounted for 43% of the variance (Figure 1), showing that *P.*
136 *aeruginosa* PA14 gene expression in the EVPL-associated biofilm may have been more
137 similar at 48 h and 7 d than 24 h. This was also observed with hierarchical clustering analysis
138 and Pearson's correlation coefficient analysis (Figure S2).

139



140 **Figure 1. Principal component analysis (PCA) plot of the *Pseudomonas aeruginosa* PA14 transcriptome,**
141 **considering all genes (n = 5829), in the *ex vivo* pig lung (EVPL) model biofilm at 24 h, 48 h, and 7 d.** RNA was
142 sequenced from the lung-associated biofilm at each time point from three repeats, from each of two
143 independent pig lungs, shown by individual data points. Each time point is shown by a different shaped data
144 point, and the individual pig lungs are shown by different colours (see key). The 95% confidence ellipses are
145 also shown.

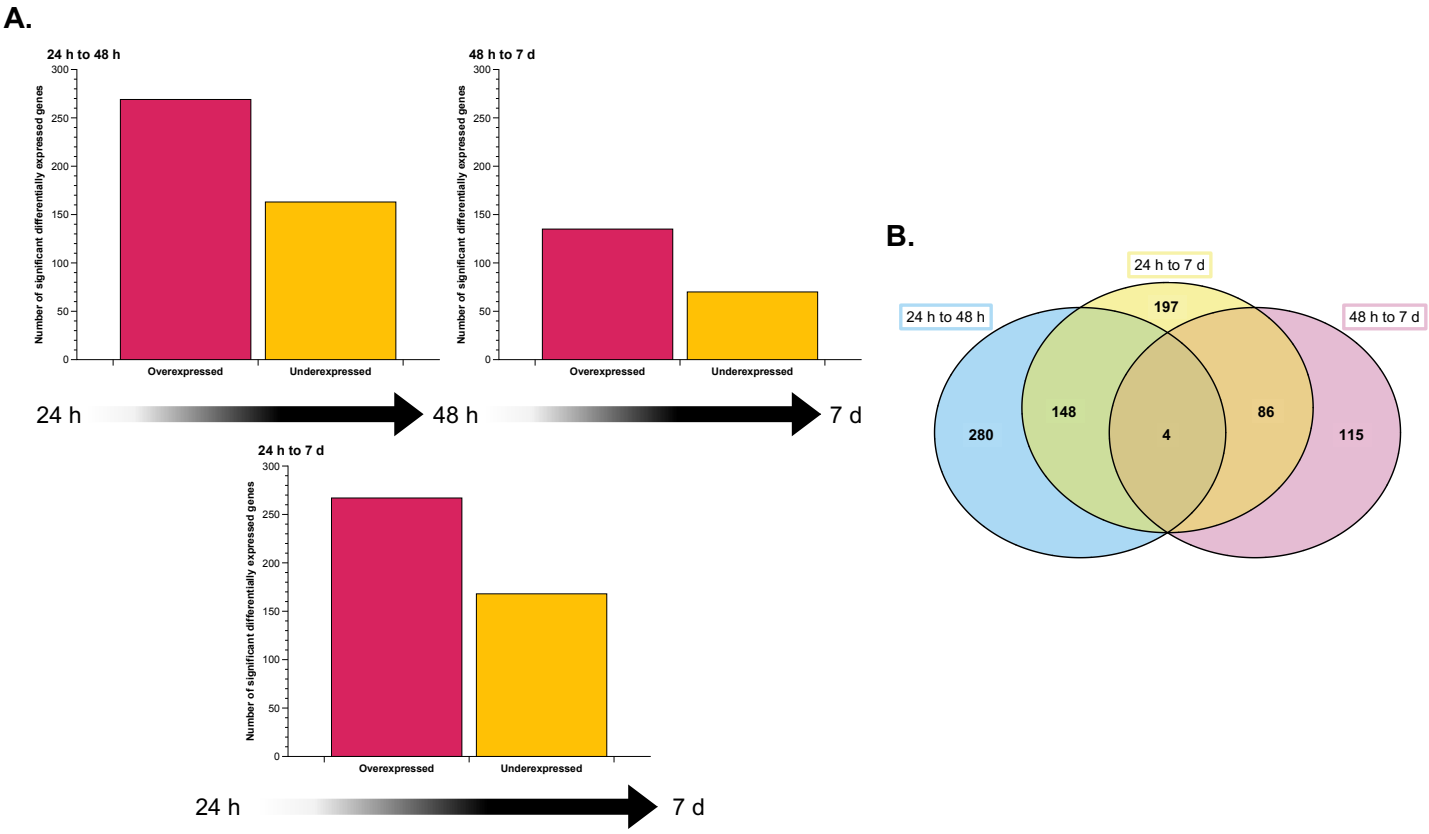
146

147 Gene expression was then compared between pairs of time points to identify genes that
148 were significantly over- or underexpressed over time. There were more significantly
149 overexpressed genes than underexpressed genes when comparing sequential time points,
150 with the greatest number of significantly overexpressed genes at 7 d compared with 24 h
151 (267 genes; Figure 2A). Furthermore, the 24 h to 48 h contrast had the greatest number of
152 unique significant differentially expressed genes (DEGs) (Figure 2B). This further indicates

153 that there was a greater difference in gene expression at 24 h compared with 48 h and 7 d,

154 than from 48 h to 7 d.

155



156 **Figure 2. The number of *Pseudomonas aeruginosa* PA14 significant differentially expressed genes (DEGs) (*P* <**

157 **0.05, $\log_2$ fold change ≥ |1.5|) in each time contrast in the *ex vivo* pig lung (EVPL) model biofilm.** RNA was

158 sequenced from three tissue-associated biofilms from each of two independent pig lungs, and differential

159 expression analysis was performed comparing three time points: 24 h, 48 h, and 7 d. **(A)** Bar charts showing

160 the number of significantly overexpressed genes (pink, left bar) and significantly underexpressed genes

161 (yellow, right bar) in each time contrast. Significant DEGs were determined at 48 h compared with 24 h (24 h

162 to 48 h), at 7 d compared with 48 h (48 h to 7 d), and at 7 d compared with 24 h (24 h to 7 d). **(B)** A Venn

163 diagram of the number of significant DEGs. The shared genes were either significantly overexpressed or

164 underexpressed in all contrasts compared. Genes that were significant in multiple contrasts but in opposing

165 directions were not considered to be shared.

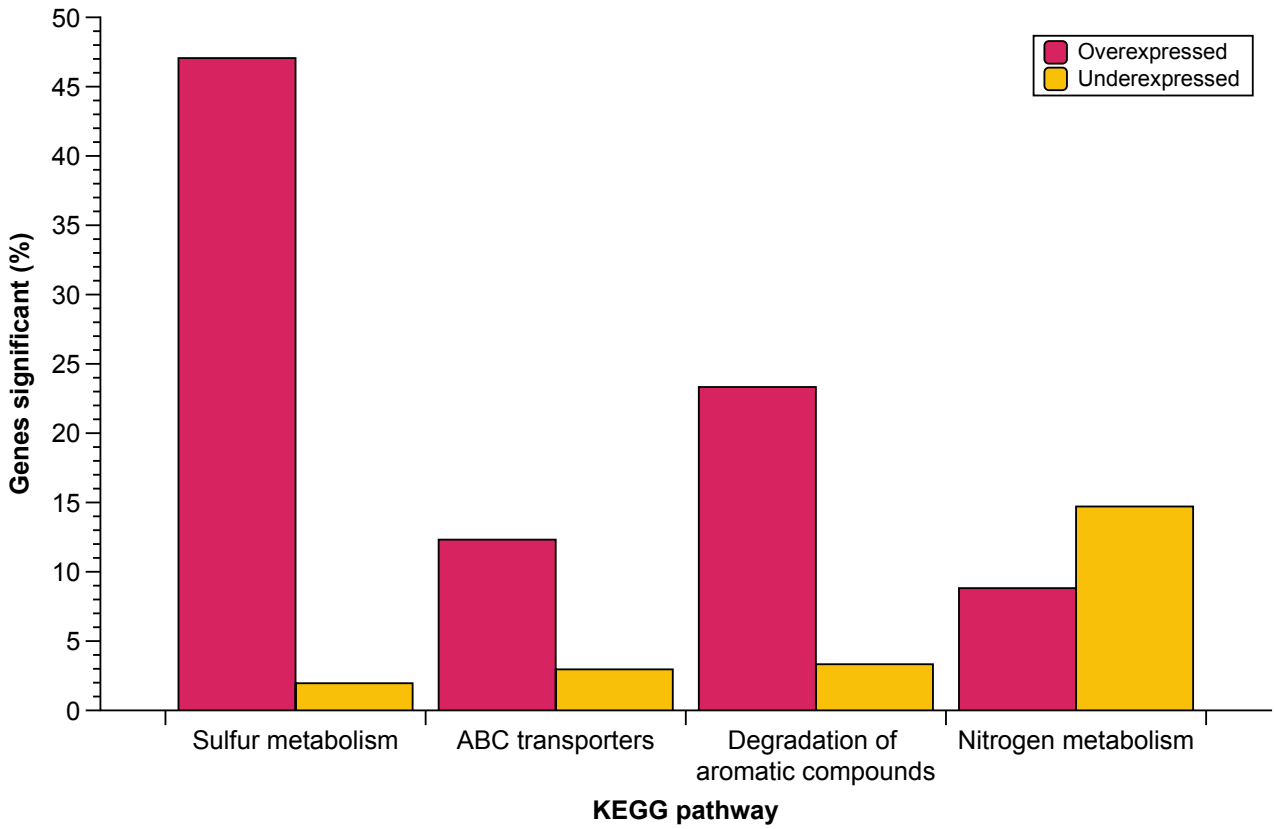
166

167 **Sulfur metabolism was upregulated from 24 h to 7 d in the EVPL-associated biofilm.**

168

169 An overview of the functional implications of the significant DEGs found was investigated
170 using KEGG enrichment analysis. The analysis identified KEGG pathways that were
171 significantly enriched ($P < 0.05$) for each time contrast. Although overall there was least
172 difference in gene expression from 48 h to 7 d (Figure 2), some KEGG pathways were found
173 to be significantly enriched (see Figure S3); these were predominantly associated with the
174 upregulation of phenazine biosynthesis. There were no *P. aeruginosa* PA14 KEGG pathways
175 significantly enriched from 24 h to 48 h, however there were four pathways significantly
176 enriched from 24 h to 7 d: sulfur metabolism, ATP-binding cassette (ABC) transporters,
177 degradation of aromatic compounds, and nitrogen metabolism. The majority of the
178 significant DEGs associated with three of these pathways were overexpressed, indicating
179 that they were upregulated over 7 d (Figure 3).

180



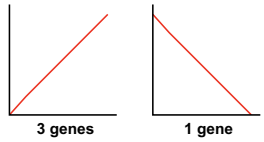
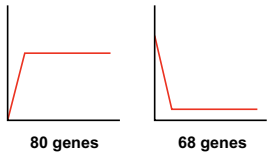
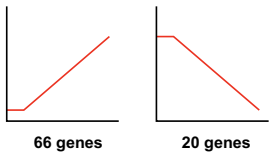
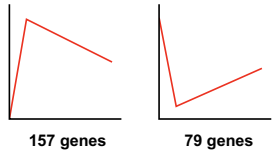
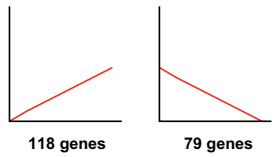
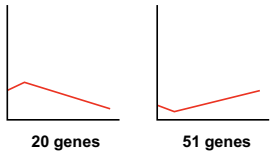
181 **Figure 3. Significantly enriched (*P* < 0.05) *Pseudomonas aeruginosa* PA14 Kyoto encyclopedia of genes and**
182 **genomes (KEGG) pathways in the *ex vivo* pig lung-associated biofilm at 7 d compared with 24 h.** Each bar
183 shows the percentage of genes associated with each pathway that were significantly differentially expressed (*P*
184 < 0.05, $\log_2$ fold change ≥ |1.5|). The overexpressed genes (left bar) and underexpressed genes (right bar) are
185 show by different colour bars (see key). *P. aeruginosa* PA14 biofilms associated with three tissue pieces from
186 each of two independent pig lungs were studied at each time point.

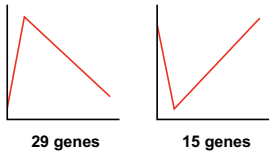
187

188 The KEGG pathway with the greatest percentage of associated genes significantly
189 differentially expressed from 24 h to 7 d was sulfur metabolism (Figure 3); 47.06% of sulfur
190 metabolism genes were significantly overexpressed in the EVPL biofilm by 7 d. This included
191 the sulfate transport genes *cysA*, *cysW*, and *cysT* (3.04, 2.87, and 3.14 $\log_2$FC respectively).
192 There was only one associated gene significantly underexpressed at 7 d: the sulfite/nitrate
193 reductase PA14_10550 (-1.56 $\log_2$FC). The majority of the significantly differentially
194 expressed ABC transporter genes were also involved in sulfur metabolism. Additionally,

195 three of the ABC transporter genes significantly overexpressed from 24 h to 7 d were part of
196 the phosphate-specific transporter PstSCAB: *pstB* (1.52 $\log_2$FC), *pstA* (1.83 $\log_2$FC), and *pstC*
197 (1.88 $\log_2$FC).
198
199 Nitrogen metabolism was the only significantly enriched KEGG pathway where more
200 significant DEGs were underexpressed than overexpressed from 24 h to 7 d (Figure 3).
201 However, it was not clear whether this pathway was upregulated or downregulated over
202 time as a similar number of genes were significantly overexpressed and underexpressed
203 (8.82% and 14.71% respectively). Expression of genes in the *P. aeruginosa* nitrogen
204 metabolism operons responsible for a shift towards anaerobic respiration in CF sputum (*nor*,
205 *nir*, *nap*, and *nos*, [21]) was investigated, but there was no clear pattern of upregulation or
206 downregulation (Table S1).
207
208 **There were seven gene expression profiles in the EVPL *P. aeruginosa* PA14 biofilm**
209 **population, associated with a number of KEGG pathways including sulfur metabolism.**
210
211 The gene expression profiles of the significant DEGs were then determined, to investigate
212 how gene expression changed over the three time points in the EVPL-associated biofilm.
213 Expression profiles were identified based on when expression changes were shared, unique,
214 or non-significant between the time point comparisons. Table 1 describes each of the seven
215 expression profiles found for *P. aeruginosa* PA14 in the EVPL-associated biofilm from 24 h to
216 48 h, to 7 d.
217

218 **Table 1. *Pseudomonas aeruginosa* PA14 gene expression profiles identified in the *ex vivo* pig lung-associated**
219 **biofilm at 24 h, 48 h, and 7 d.** Differential expression analysis was performed using three repeats from each of
220 two independent pig lungs. Contrasts were performed at 48 h compared with 24 h, 7 d compared with 48 h,
221 and 7 d compared with 24 h. The changes in expression of each significant differentially expressed gene found
222 in any contrast was investigated across all three time points, to determine the expression changes over time.
223 The seven different expression profiles found were separated into groups, described in the table. The graphical
224 representations demonstrate the changes in expression of genes over the three time points. Below each graph
225 is the number of significant differentially expressed genes found to follow that expression profile in the lung-
226 associated *P. aeruginosa* PA14 biofilm.

Profile number	Gene expression profile	Graphical representation
1	Significant change in expression 24 h to 48 h, then a significant change in the **same** direction 48 h to 7 d. Results in significant change 24 h to 7 d.	
2	Significant change in expression 24 h to 48 h, which is **maintained** 48 h to 7 d (no significant change). Results in significant change 24 h to 7 d.	
3	No significant change in expression 24 h to 48 h, then a significant change 48 h to 7 d. Results in significant change 24 h to 7 d.	
4	Significant change in expression 24 h to 48 h, then a small but not significant change in the **opposite** direction 48 h to 7 d. Results in no significant change 24 h to 7 d.	
5	Small but not significant change in expression 24 h to 48 h, then a small but not significant change in the **same** direction 48 h to 7 d. Results in overall significant change 24 h to 7 d.	
6	Small but not significant change in expression 24 h to 48 h, then a significant change in the **opposite** direction 48 h to 7 d. Results in no significant change 24 h to 7 d.	

7	Significant change in expression 24 h to 48 h, then a significant change in the **opposite** direction 48 h to 7 d. Results in no significant change 24 h to 7 d.	

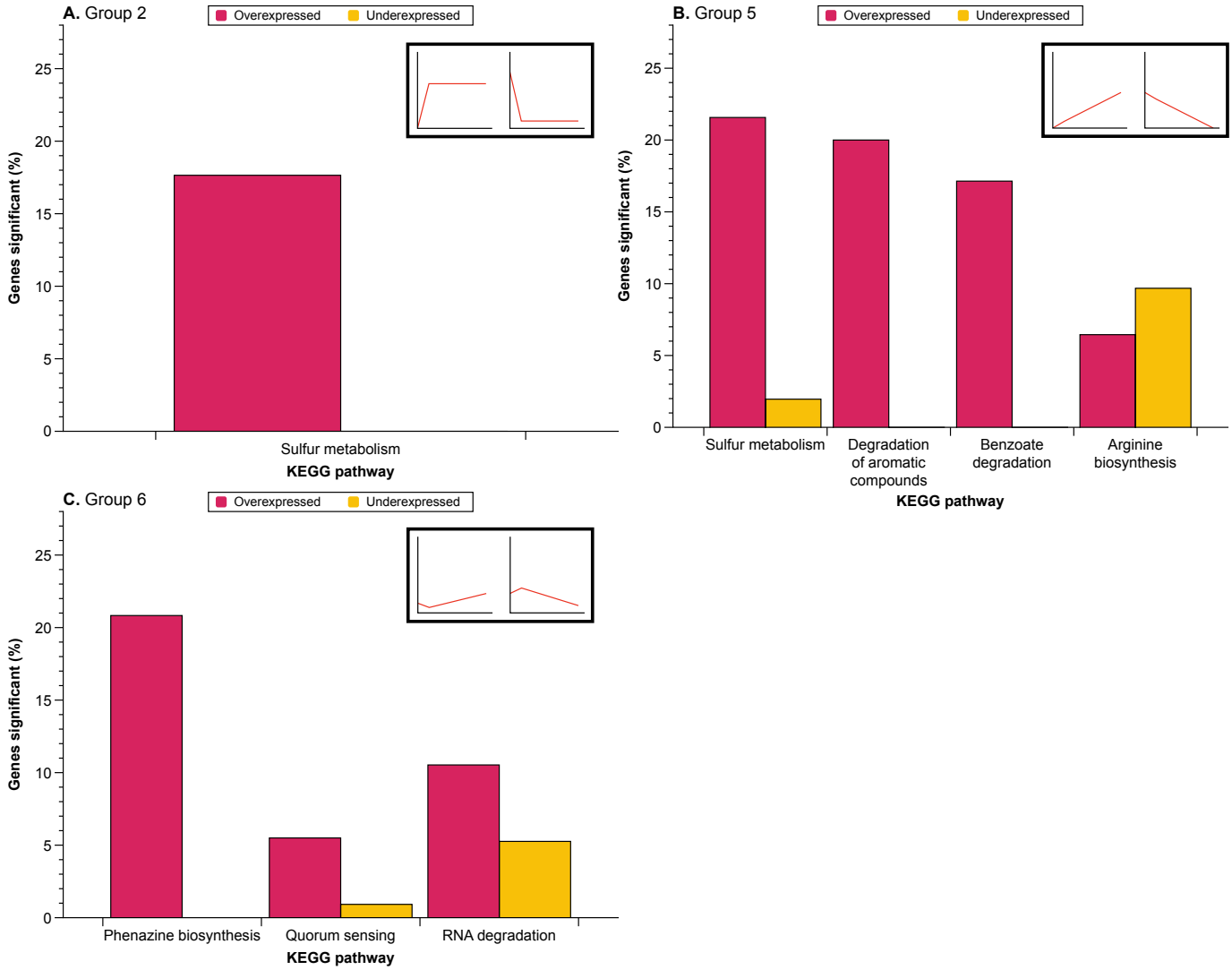
There were four genes that were significantly differentially expressed in the same direction from 24 h to 48 h, and 48 h to 7 d (Figure 2B), which followed expression profile 1 (Table 1). Further investigation of these genes did not indicate any specific pathways following this steep increase or decrease in expression (Table S2). The greatest number of significant DEGs followed expression profile 4 (Table 1). These genes were only significantly differentially expressed 24 h to 48 h, then a non-significant change in expression in the opposite direction 48 h to 7 d resulted in no overall significant difference 24 h to 7 d (Table 1).

KEGG enrichment analysis was performed for each of the gene expression profiles in the *P. aeruginosa* PA14 EVPL-associated biofilm populations. Significantly enriched KEGG pathways ($P < 0.05$) were found for three of the profiles (Figure 4). Consistent with the previous finding, sulfur metabolism was found to be significantly enriched and the associated genes followed two expression profiles: 2 and 5. These genes were overexpressed following both expression profiles; either upregulation from 24h to 48 h that was maintained to 7 d (Figure 2A), or a gradual upregulation (Figure 2B). The degradation of aromatic compounds and benzoate degradation KEGG pathways were similarly significantly enriched following expression profile 5 (Figure 4B). All significant DEGs in the degradation of aromatic compounds pathway were part of the benzoate degradation pathway. Arginine biosynthesis was also significantly enriched following profile 5 (Figure 4B); two of the significantly

247 associated underexpressed genes were arginine fermentation enzymes [22]: *arcB* (-2.25

248 $\log_2$FC) and *arcC* (-2.16 $\log_2$FC), which have been shown to be induced under stress [23, 24].

249



250 **Figure 4. Significantly enriched (*P* < 0.05) *Pseudomonas aeruginosa* PA14 Kyoto encyclopedia of genes and**

251 **genomes (KEGG) pathways found for gene expression profiles in the *ex vivo* pig lung-associated biofilm over**

252 **24h, 48 h, and 7 d.** The expression of significantly differentially expressed genes over the three time points

253 was investigated and separated into seven different profiles, three of which were found to involve sufficient

254 genes associated with KEGG pathways for pathways to be significantly enriched. The bars show the percentage

255 of significant differentially expressed genes associated with each enriched pathway ($P < 0.05$, $\log_2$ fold change

256 ≥ |1.5|); those overexpressed (left bar) and underexpressed (right bar) are shown by different colours (see

257 key). The graphical representation of the gene expression profile is shown in the top right of each graph; the

258 left graph shows the profile of overexpressed genes, and the right graph shows the profile of underexpressed
259 genes. Three repeats from each of two independent pig lungs were studied at each time point. **(A)** Significantly
260 enriched KEGG pathway following expression profile 2, where there was a significant change 24 h to 48 h,
261 which was maintained 48 h to 7 d resulting in a significant change 24 h to 7 d. **(B)** Significantly enriched KEGG
262 pathways following profile 5, where there was a significant change in expression in the direction specified 24 h
263 to 7 d, caused by non-significant changes in the same direction 24 h to 48 h, and 48 h to 7 d. **(C)** Significantly
264 enriched KEGG pathways following profile 6, where there was a significant change in the direction specified at
265 7 d compared with 48 h.
266
267 Gene expression profile 6 was composed of genes that were only significantly differentially
268 expressed from 48 h to 7 d (Table 1). Three KEGG pathways were significantly enriched
269 following this expression profile, including phenazine biosynthesis and QS which were
270 upregulated (Figure 4C). All the significant differentially expressed genes associated with
271 phenazine biosynthesis were also associated with the QS pathway.
272
273 ***P. aeruginosa* PA14 expression of genes associated with antimicrobial resistance was**
274 **reduced over time in the EVPL biofilm but tolerance to colistin and polymyxin B increased.**
275
276 We previously found significant differential expression of AMR genes when *P. aeruginosa*
277 PA14 was grown in the EVPL model compared with SCFM *in vitro*, at both 24 h and 48 h [19].
278 We were therefore interested in investigating whether changes in AMR gene expression
279 occurred in the EVPL-associated biofilm over 7 d infection. Analysis of AMR gene expression
280 found three genes significantly underexpressed at either 48 h or 7 d compared with 24 h
281 (Table 2), and no genes significantly overexpressed. The full AMR gene list studied is shown
282 in Table S3. The *brc-1* gene, which encodes a major facilitator superfamily (MFS) transporter

283 associated with bicyclomycin resistance [25], was significantly underexpressed from 24 h to
284 7 d. The other two significant DEGs found were significantly underexpressed from 24 h to 48
285 h. This included *mexG* and *arnA*, which encode a transmembrane protein associated with
286 multiple efflux pumps and a bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-
287 4-deoxy-L-arabinose formyltransferase [3], respectively. More specifically, the enzyme
288 encoded by *arnA* is involved in lipid A modifications, linked to cationic antimicrobial peptide
289 (CAMP) resistance [26, 27].
290

291 **Table 2. *Pseudomonas aeruginosa* PA14 antibiotic resistance associated genes found to be significantly**
292 **differentially expressed in at least one contrast in the *ex vivo* pig lung-associated biofilm between 24 h, 48 h,**
293 **and 7 d.** Differential expression analysis was performed on three repeats from each of two independent pig
294 lungs at 48 h compared with 24 h, 7 d compared with 48 h, and 7 d compared with 24 h. The gene expression
295 group denotes the expression profile the gene belonged to (see Table 1). The $\log_2$ fold change ($\log_2$FC) in the
296 contrast where the gene was significantly differentially expressed is shown. A gene was considered
297 significantly differentially expressed if $\log_2$FC ≥ |1.5| and $P < 0.05$.

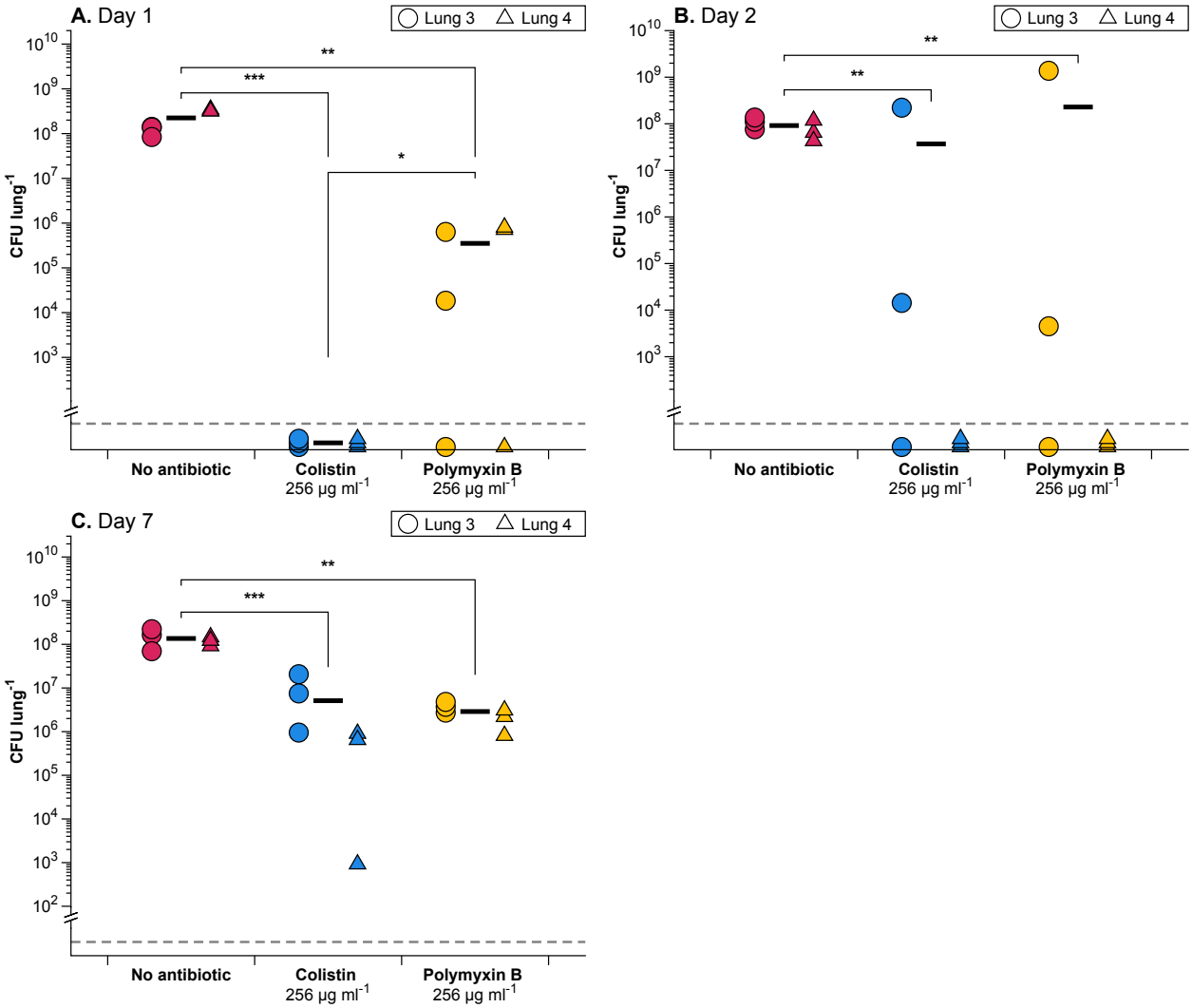
Locus tag	Gene name	Gene product	Gene expression group	Time: Log_2FC
PA14_09540	*mexG*	Transmembrane protein	4	24 h to 48 h: -1.52
PA14_18350	*arnA*	Bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	4	24 h to 48 h: -1.59
PA14_10470	*bcr-1*	MFS transporter	5	24 h to 7 d: -2.49

298

299 To determine how AMR gene expression compared to phenotype, *P. aeruginosa* PA14 was
300 grown as a biofilm on the EVPL tissue for 24 h, 48 h or 7 d then exposed to one of two
301 clinically relevant polymyxins, polymyxin B or colistin, for a further 24 h. The CFU lung^{-1} was

302 determined and compared with PA14 EVPL biofilms treated with phosphate-buffered saline
303 (PBS) for the second 24 h incubation as a negative control (Figure 5). Samples initially grown
304 for 24 h prior to treatment did not exhibit any growth when exposed to 256 µg ml^{-1} colistin
305 (Figure 5A). After 48 h initial biofilm growth, some samples were able to survive following
306 colistin exposure (Figure 5B). However, after 7 d growth followed by colistin exposure, the
307 *P. aeruginosa* PA14 biofilm populations were able to tolerate 256 µg ml^{-1} colistin and
308 maintain a CFU $lung^{-1}$ comparable to the untreated samples (Figure 5C). A similar pattern
309 was observed following 256 µg ml^{-1} polymyxin B treatment; PA14 biofilm tolerance
310 increased with time following initial infection (Figure 5), and 7 d biofilms were consistently
311 able to survive treatment (Figure 5C). The pattern of increased polymyxin tolerance with
312 biofilm age is a contrast to the reduced expression of AMR-associated genes observed,
313 suggesting indirect factors may be involved.

314



315 **Figure 5. The colony forming units per lung tissue piece (CFU lung^{-1}) for the *Pseudomonas aeruginosa* PA14**

316 **tissue-associated biofilm of different ages following no antibiotic treatment, treatment with 256 µg ml^{-1}**

317 **colistin, or 256 µg ml^{-1} polymyxin B.** Three replicates from each of two independent pig lungs, shown by

318 different shaped data points (see key), were used. The *P. aeruginosa* PA14 biofilms were grown in the *ex vivo*

319 pig lung (EVPL) model for **(A)** 24 h **(B)** 48 h **(C)** 7 d, then exposed to either phosphate-buffered saline (PBS) as a

320 negative control (no antibiotic; pink), 256 µg ml^{-1} colistin (blue), or 256 µg ml^{-1} polymyxin B (yellow) for a

321 further 24 h. The CFU lung^{-1} was then determined and is shown on the graphs, data points below the dashed

322 horizontal line represent samples where no CFU were detected. The asterisks represent a significant difference

323 between treatments determined using post-hoc Tukey HSD tests following ANOVA (* = $P < 0.05$). **(A)** Day 1

324 ANOVA: treatment $F_{2,12} = 30.32$, $P < 0.01$; lung $F_{1,12} = 0.15$, $P = 0.71$; interaction $F_{2,12} = 0.04$, $P = 0.96$. **(B)** Day 2

325 ANOVA: treatment $F_{2,12} = 10.53$, $P < 0.01$; lung $F_{1,12} = 5.76$, $P = 0.03$; interaction $F_{2,12} = 1.26$, $P = 0.32$. **(C)** Day 7

326 ANOVA: treatment $F_{2,12} = 14.51$, $P < 0.01$; lung $F_{1,12} = 4.12$, $P = 0.07$; interaction $F_{2,12} = 2.30$, $P = 0.14$.

327

328 **There was significantly reduced expression of Pel biosynthesis genes from 24 h to 48 h in**

329 **the EVPL-associated biofilm, but a dense, structured biofilm was clear at 48 h.**

330

331 QS and biofilm formation pathways were then investigated, as both are involved in the

332 establishment of a chronic *P. aeruginosa* infection in the CF lung and may have been

333 associated with the increased antibiotic tolerance observed. As shown in Figure 6, there was

334 no overall difference in QS gene expression from 24 h to 48 h. This was similarly observed

335 from 48 h to 7 d (Figure S4), and from 24 h to 7 d (Figure S5). Other genes associated with *P.*

336 *aeruginosa* PA14 virulence factor production were also investigated, including those

337 involved in motility, type III secretion, type VI secretion, and alginate biosynthesis. However,

338 there was no significant differential expression found in the lung-associated biofilm over the

339 three time points for any of these processes.

340

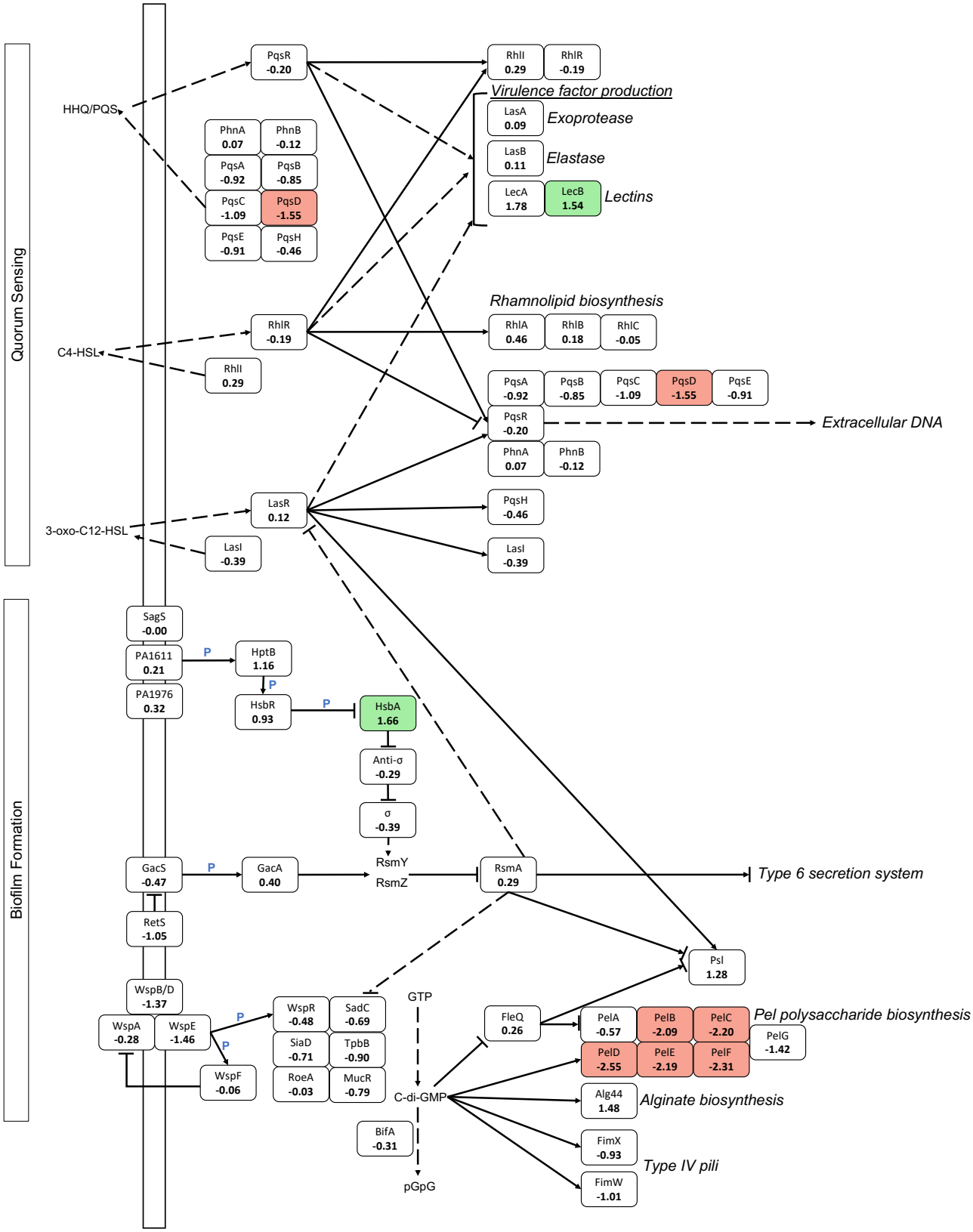


Figure 6. Diagram of the gene expression changes in the *Pseudomonas aeruginosa* PA14 Kyoto encyclopedia of genes and genomes (KEGG) biofilm formation and quorum sensing (QS) pathways in the *ex vivo* pig lung-associated biofilm from 24 h to 48 h. Three repeats from each of two independent pig lungs were studied at each time point. The diagram shows all genes involved in each pathway, including associated signalling

345 pathways and the Pel exopolysaccharide biosynthesis pathway. The $\log_2$ fold change value from 24 h to 48 h is
346 shown below each gene name. The genes shown in red were significantly underexpressed, and genes shown in
347 green were significantly overexpressed (significance: $P < 0.05$, $\log_2$ fold change ≥ |1.5|). The blocked arrows
348 represent gene activation, and the dashed arrows show indirect gene activation. The solid, blocked lines
349 (ending with |, not an arrow) show inhibition, and the dashed, blocked lines show indirect inhibition. The blue
350 'P' represents phosphorylation.

351

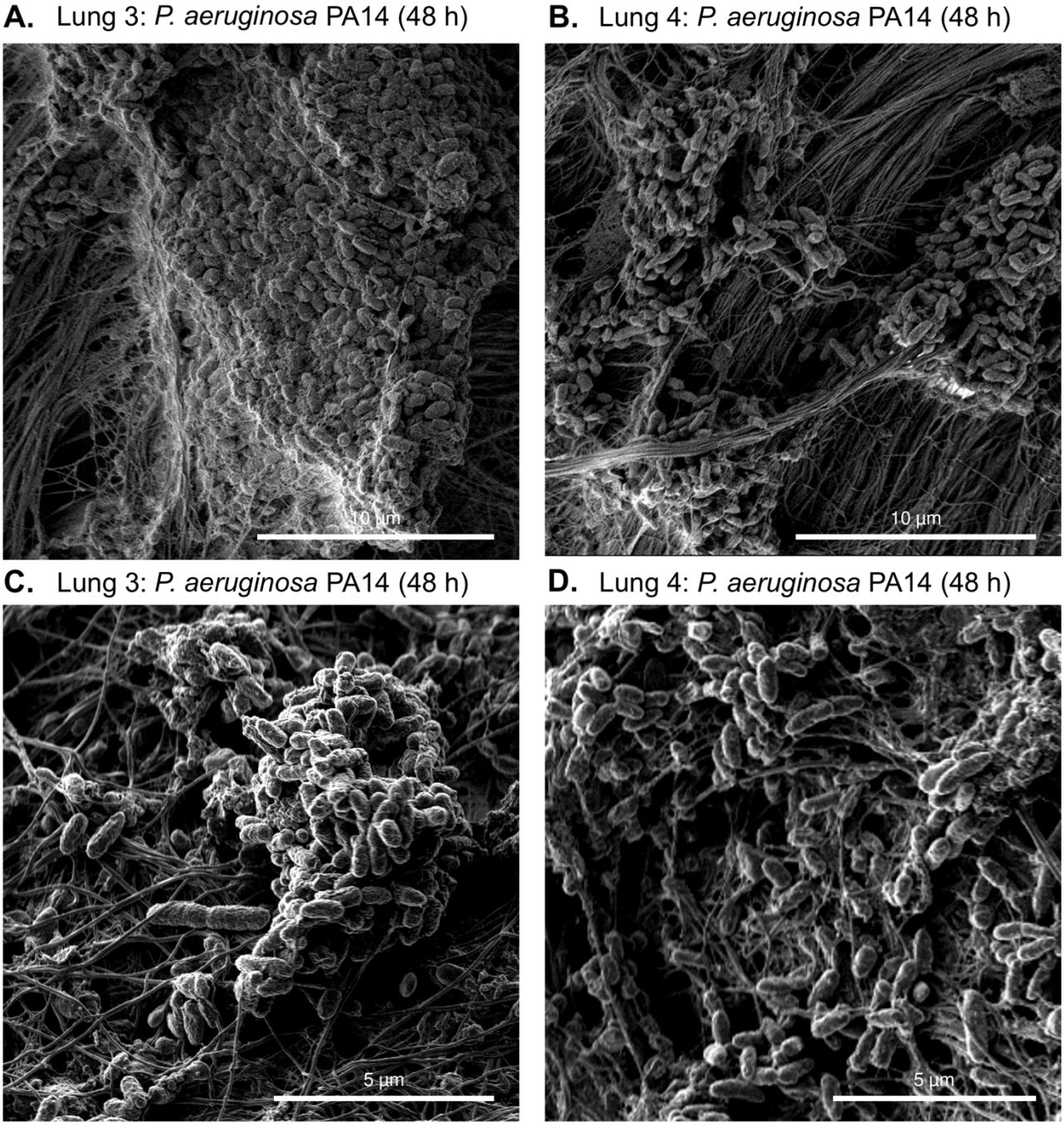
352 There were only two genes found to be significantly overexpressed from 24 h to 48 h in
353 these pathways, including *lecB* (Figure 6). LecB is the second *P. aeruginosa* internal lectin
354 (PA-IIL) that has been shown to act as a biofilm matrix protein, and is associated with
355 increased cell and exopolysaccharide retention in the biofilm [28]. The anti-sigma factor
356 antagonist HsbA, associated with RpoS regulation [29], was also found to be significantly
357 overexpressed from 24 h to 48 h. However, there were no other genes in this pathway
358 found to be significant differentially expressed (Figure 6), indicating that this differential
359 expression may not have had an effect. In contrast, five out of the seven genes in the
360 operon for biosynthesis of the biofilm exopolysaccharide Pel were found to be significantly
361 underexpressed from 24 h to 48 h (Figure 6). The other two genes were also
362 underexpressed but below the $\log_2$FC threshold considered significant. This downregulation
363 was maintained for the rest of the 7 d, with all genes in the operon significantly
364 underexpressed at 7 d compared with 24 h (Figure S5).

365

366 Scanning electron microscopy (SEM) was then performed to visualise the *P. aeruginosa*
367 PA14 biofilm formed on the surface of the EVPL tissue at 24 h, 48 h, and 7 d, to determine

368 the phenotypic implications of the differential expression. Uninfected tissue was also
369 imaged as a negative control (Figure S6). A biofilm could not be imaged at 24 h, however
370 there was a dense, structured *P. aeruginosa* PA14 biofilm clear at 48 h despite significantly
371 reduced expression of genes for biosynthesis of the biofilm exopolysaccharide Pel (Figure 7).
372 This was consistent with previous work performed using the EVPL model [20]. The biofilm
373 population was maintained at 7 d, where the *P. aeruginosa* PA14 cells remained clearly
374 visible (Figure S7).

375



376 **Figure 7. Scanning electron micrographs of *Pseudomonas aeruginosa* PA14 biofilms associated with *ex vivo***

377 **pig lung (EVPL) bronchiolar tissue 48 h after infection.** Images were taken from biofilms formed on tissue

378 from two independent pig lungs: **(A, C)** lung 3 **(B, D)** lung 4. Scale bars are shown. Images were taken using a

379 Zeiss Gemini electron microscope with an InLens detector, at 1kV, and analysed using Omero5.6 [30]

380

381 **Discussion**

382

383 Extended analysis of an RNA-sequencing dataset [19] identified distinct transcriptomes of
384 the *P. aeruginosa* PA14 biofilm population associated with EVPL bronchiolar tissue over 7 d
385 infection. Differential expression analysis was performed comparing gene expression at 24
386 h, 48 h, and 7 d, and no significant changes in expression of key regulatory genes were
387 found. There was however upregulation of a number of non-stress related KEGG pathways,
388 and the majority of significant DEGs were overexpressed. This indicated that the biofilm was
389 maintained for 7 d and the population was not dying. The greatest difference in expression
390 was between 24 h and the two later time points: 48 h and 7 d. SEM showed that a dense
391 biofilm had formed by 48 h, with *P. aeruginosa* cells at 7 d that did not exhibit visible
392 indications of stress. These findings demonstrate that the EVPL model provides an
393 environment for *P. aeruginosa* biofilm formation that not only phenotypically exhibits
394 characteristics of a mature biofilm at 7 d as previously described [20], but also maintains the
395 *P. aeruginosa* PA14 population for 7 d infection.

396

397 Sulfur metabolism was found to have the highest percentage of significant DEGs associated
398 of all the significantly enriched KEGG pathways over time. It was significantly upregulated
399 from 24 h to 7 d, and the associated genes followed two different expression profiles. There
400 was with a gradual increase in expression from 24 h to 7 d, or a significant increase 24 h to
401 48 h that was maintained up to 7 d. More specifically, all genes in the sulfur transporter
402 operon associated with cysteine biosynthesis: *cysTWA*, were found to be significantly
403 overexpressed 24 h to 7 d. In *E. coli,* upregulation of this operon has been linked to sulfate
404 starvation [31]. However, the role of these genes in *P. aeruginosa* biofilms is less

405 understood. Investigation of the sulfate starvation response in *P. aeruginosa* PAO1 and a
406 cystic fibrosis clinical isolate found genes involved in cysteine biosynthesis, including
407 *cysTWA*, were only weakly upregulated when sulfate was limited [32]. As outlined, *P.*
408 *aeruginosa* PA14 upregulation of sulfur metabolism in SCFM has actually been linked to a
409 shift towards anaerobic respiration [11]. There have also been links made with iron
410 starvation and sulfur metabolism. In fact, during iron starvation proteins involved in cysteine
411 biosynthesis and sulfur assimilation are reduced [33]. Therefore, upregulation of sulfur
412 metabolism in the EVPL biofilm may have been a result of a change of lifestyle, such as
413 anaerobic respiration or iron availability, rather than sulfur starvation. Thus, these findings
414 may further support the progression of *P. aeruginosa* biofilm infection in the EVPL model as
415 capturing the lifestyle changes observed in CF. However, sulfur and oxygen availability in the
416 EVPL biofilm should be determined in the future to confirm this hypothesis.
417
418 Among the pathways upregulated was also phenazine biosynthesis; the associated genes
419 were significantly overexpressed from 48 h to 7 d. More specifically, genes involved in
420 biosynthesis of the phenazine virulence factor pyocyanin were significantly overexpressed.
421 Pyocyanin acts as a signalling molecule for *P. aeruginosa* PA14 by upregulating efflux pump
422 genes, and genes involved in iron acquisition and redox. Additionally, when *P. aeruginosa*
423 PA14 biofilms have been grown in flow cells, phenazines, specifically pyocyanin, have been
424 shown to play a role in the formation of a biofilm with defined architecture, and phenazine-
425 deficient mutants were unable to form a structured biofilm [34]. Therefore, upregulation of
426 this pathway from 48 h to 7 d in the PA14 EVPL-associated biofilm may be linked to biofilm
427 maturation once it has formed at 48 h, leading to the *in vivo*-like architecture previously
428 observed at 7 d [20].

429

430 Pyocyanin is also believed to link the *Pseudomonas* quinolone signal (PQS) QS system with
431 expression of the efflux pump MexGHI-OpmD [35], which we previously found to be
432 significantly underexpressed in the PA14 EVPL biofilm compared with *in vitro* SCFM growth
433 at 48 h [19]. All three *P. aeruginosa* QS systems are involved in the regulation of phenazine
434 biosynthesis operons, including production of phenazine-1-carboxylic acid (PCA) and
435 pyocyanin [36]. The upregulation of phenazine biosynthesis thus indicates that there were
436 significant changes in expression of QS-regulated pathways over time in the EVPL biofilm.
437 Following this, and our previous work indicating that the EVPL biofilm may more accurately
438 recapitulate *P. aeruginosa* QS gene expression in CF sputum than other *in vitro* models [19],
439 *P. aeruginosa* PA14 changes in expression of genes in the three QS pathways was
440 investigated: LasI/R, RhlI/R, and PQS. There were no significant differences found in the
441 expression of genes in these pathways for any of the time contrasts in the EVPL biofilm,
442 suggesting changes in the QS systems did not occur over 7 d. Other regulatory pathways
443 involved in lifestyle changes, including the switch from acute to chronic infection, were also
444 investigated however no clear differences in expression were found either.

445

446 There were also no significant changes in virulence factor gene expression found. It is known
447 that as part of adaptation to the CF lung and formation of a chronic infection, mutations in
448 *P. aeruginosa* virulence pathways are selected for and virulence factor production is
449 significantly reduced [37]. It is hypothesised that *P. aeruginosa* may have already switched
450 off virulence factor production by 24 h when it was forming the established biofilm
451 visualised at 48 h, hence no change in gene expression was observed. This is supported by
452 the findings in our previous study, in which we found two of the major protease-encoding

453 genes *lasA* and *lasB* were significantly underexpressed in the EVPL-associated biofilm
454 compared with SCFM *in vitro* growth at both 24 h and 48 h [19]. This may also explain why
455 no significant differential expression was observed in regulatory pathways including QS.
456 Future work looking at the accumulation of mutations over 7 d in the EVPL-associated *P.*
457 *aeruginosa* biofilm is required to confirm this hypothesis.
458
459 There were however significant changes in expression of biofilm-associated genes found in
460 the EVPL PA14 biofilm population over 7 d. Pel biosynthesis was significantly downregulated
461 from 24 h to 48 h, then maintained at the same expression level from 48 h to 7 d, which
462 resulted in significant downregulation from 24 h to 7 d. This suggested that *P. aeruginosa*
463 PA14 production of Pel was reduced after 24 h of infection, then kept at the same level from
464 48 h onwards. Pel is a biofilm exopolysaccharide that is essential to biofilm development by
465 *P. aeruginosa* PA14 and is only expressed during biofilm growth [38, 39]. It must be
466 considered that Pel was significantly underexpressed in the EVPL biofilm at 48 h and 7 d
467 compared with 24 h. Thus, our results do not indicate that there was not any Pel being
468 produced at the later time points, just less than the initial stage of infection in the EVPL
469 model. This may have been caused by upregulation of Pel biosynthesis as the biofilm was
470 established at 24 h, which was reduced once the biofilm was formed at 48 h, and then
471 maintained at this lower level over the remaining 5 d. This hypothesis was supported by
472 SEM images of the biofilm, which showed a dense, structured *P. aeruginosa* biofilm already
473 present at 48 h.
474
475 Taking this into account, following formation of a biofilm, gene expression analysis indicated
476 that the focus may have shifted from production of the biofilm matrix to biofilm

477 maintenance and maturation from 48 h to 7 d. Significant upregulation of two lectin genes
478 was also observed in the lung-associated biofilm from 24 h to 7 d, and from 24 h to 48 h.
479 Both have been associated with a mature biofilm; *lecA* is indicated to play a role in the
480 formation of a mature biofilm architecture [40], and *lecB* has been shown to be involved in
481 increased cell and exopolysaccharide retention in biofilms [28]. This retention may also have
482 been associated with the reduction in Pel production, as less new Pel would be required if
483 the Pel already produced was being retained. This proposed maintenance of a mature
484 biofilm structure was also accompanied by increased antimicrobial tolerance.
485
486 In our first study of *P. aeruginosa* PA14 gene expression in the EVPL model compared with *in*
487 *vitro* SCFM growth, we found that the EVPL growth environments cued differential
488 expression of a number of AMR genes despite no antibiotic exposure, at both 24 h and 48 h
489 [19]. However only three genes predicted to be involved in AMR were found to be
490 differentially expressed over 7 d in the EVPL biofilm, suggesting that this differential
491 expression of resistance-associated genes cued by the environment is unlikely to be
492 dynamic and remains consistent throughout 7 d infection.
493
494 The significantly differentially expressed AMR genes that were found included significant
495 reduction in expression of two individual antibiotic resistance genes from 24 h to 48 h: *arnA*
496 and *bcr-1*, associated with resistance to CAMPs [27] and bicyclomycin [25] respectively.
497 However, the *P. aeruginosa* PA14 biofilm population grown for 7 d in the EVPL model was
498 able to tolerate treatment with 256 µg ml^{-1} colistin or 256 µg ml^{-1} polymyxin B at 7 d,
499 despite not being able to following 24 h growth. Thus, there were other mechanisms
500 involved by 7 d that resulted in increased *P. aeruginosa* polymyxin tolerance. It is

501 hypothesised that the formation of a biofilm, of which the matrix facilitates antibiotic
502 resistance [4], may have resulted in other mechanisms of antibiotic resistance being
503 downregulated as they were not required. This would provide explanation as to why
504 expression of AMR genes was reduced once a biofilm had formed, and the matrix was no
505 longer being rapidly produced. This finding would further support the use of biofilm
506 disrupters to improve treatment regimens, as the *P. aeruginosa* cells within the biofilm may
507 not be expressing genes associated with antibiotic resistance and are thus more susceptible
508 to treatments.
509
510 However, it must be considered that there were other mechanisms of antibiotic resistance
511 that were found to be significantly overexpressed in the EVPL biofilm, which are associated
512 with potential environmental pressures. Phosphate transporters were significantly
513 overexpressed from 24 h to 7 d, indicative of phosphate stress. Under phosphate stress *P.*
514 *aeruginosa* has been shown to undergo lipid modifications that confer resistance to
515 polymyxin B. There is a transition from phospholipids to glycolipids, which confer protection
516 against polymyxin B [41]. Therefore, this differential expression may also be responsible, or
517 involved in, the increased antibiotic tolerance observed. Future investigation of *P.*
518 *aeruginosa* lipid modifications in the EVPL model environment would provide further
519 insight. This analysis has however indicated that these pathways may be induced, thus
520 further understanding of lipid modifications cued by the EVPL model may highlight novel
521 treatment targets.
522
523 In summary, we have demonstrated that there are changes in the *P. aeruginosa* lung-
524 associated biofilm population in the EVPL model over 7 d, however the model is able to

525 maintain the population for this time with no obvious indicators of stress. We have
526 identified an increase in polymyxin tolerance that is accompanied by an upregulation in
527 sulfur metabolism, and maintenance of a structured biofilm, thus providing further insight
528 into *P. aeruginosa* antibiotic tolerance in CF. Introduction of other factors that influence *P.*
529 *aeruginosa* gene expression in the CF lung into the EVPL model, such as other microbes,
530 may reveal other changes in the biofilm population over time that facilitate survival in these
531 conditions. Further understanding of these mechanisms will provide better understanding
532 as to how to eradicate or prevent chronic *P. aeruginosa* infections.
533

534 ## Materials and Methods

535

536 **Bacterial strain.** The *Pseudomonas aeruginosa* PA14 wild type strain used for this work was
537 originally from the University of Washington [42, 43], and was grown on Luria-Bertani (LB)
538 agar (Melford Laboratories) for 24 h at 37 °C prior to all experiments.
539

540 **Synthetic cystic fibrosis sputum medium.** Synthetic cystic fibrosis sputum medium (SCFM)
541 was made based on the original protocol [44]. Glucose was substituted with deionised water
542 as previous work using the EVPL model demonstrated that in the presence of lung tissue,
543 the absence of glucose did not affect *P. aeruginosa* growth but did facilitate endogenous
544 lung bacterial species growth [45].
545

546 **EVPL dissection and infection.** Pig lung bronchiolar tissue was dissected and subsequently
547 infected following the previously published method [19, 20, 46]. Two independent lungs
548 were used per experiment, and three individual tissue pieces per lung. All lungs used for this

549 work were provided by Steve Quigley and Sons Butchers Ltd. (Cubbington, United Kingdom),
550 and were dissected and infected on the day of arrival from the abattoir.
551
552 **EVPL antibiotic treatment.** Two different antibiotics were tested: colistin and polymyxin B,
553 both prepared as master stocks as follows; colistin (Acros Organics) was prepared to 10 mg
554 ml^{-1} in dH_2O, and polymyxin B (Sigma-Aldrich) was prepared to 50 mg ml^{-1} in dH_2O. The
555 master stocks of each antibiotic were further diluted in SCFM, in 15 ml Falcon tubes, to the
556 desired concentration of 256 µg ml^{-1} for use with the EVPL model.
557
558 The EVPL grown biofilms were treated with each antibiotic as previously described [46].
559 Following incubation at 37 °C for either 24 h, 48 h, or 7 d, *P. aeruginosa* PA14 infected pig
560 lung tissue pieces were removed from the individual wells of a 24-well plate using flame
561 sterilised forceps. Each tissue piece was washed in 500 µl PBS in individual wells of a 48-well
562 plate to remove non-biofilm associated bacterial cells. The tissue pieces were then
563 transferred to individual wells of a 48-well plate, each containing 500 µl of either 256 µg ml^{-1}
564 colistin or 256 µg ml^{-1} polymyxin B. A replicate control, non-antibiotic treated set of tissue
565 pieces from each lung per experiment, were transferred to individual wells of a 48-well plate
566 each containing 500 µl PBS. All plates were covered with a UV sterilised Breathe-Easier®
567 sealing membrane (Diversified Biotech), and incubated stationary at 37 °C for 24 h.
568
569 **EVPL biofilm recovery and determination of bacterial load.** Bacteria was recovered from
570 the EVPL tissue pieces, and the bacterial load determined following the published method
571 [19, 20, 46]. Briefly, following the final incubation for both RNA extractions and antibiotic
572 treatments, tissue pieces were transiently washed in 500 µl PBS in individual wells of a 48-

573 well plate to remove non-biofilm bacterial cells. Each tissue piece was then transferred into
574 individual, sterile homogenisation tubes (Fisherbrand) containing 1 g of 2.38 mm metal
575 beads (Fisher Scientific) and 1 ml PBS. The tissue-containing bead tubes were placed in a
576 FastPrep-24 5G (MP Biomedicals), and bead beaten for 40 s at 4 m s^{-1} to recover bacterial
577 cells from the lung-associated biofilm.
578
579 The lung homogenate was then used to determine the biofilm bacterial load associated with
580 the EVPL tissue. An aliquot of each homogenate was serially dilute in PBS in a 96-well plate
581 to final volumes of 200 µl. The dilutions were then plated in triplicate on LB agar and
582 incubated overnight at 37 °C. The number of colonies of the two highest dilutions that were
583 countable were determined and used to calculate the colony forming units (CFU) $lung^{-1}$ for
584 each sample.
585
586 **RNA sequencing work.** All RNA samples used for this work have been published as part of
587 another analysis, and thus the same protocol was followed for extraction, precipitation,
588 DNA removal, and sequencing [19]. In the initial publication, differential expression in the
589 EVPL biofilm compared with the lung-associated planktonic growth in surrounding SCFM
590 media, and *in vitro* SCFM planktonic cultures was explored but differential expression across
591 the time course of infection in the biofilm populations was not investigated.
592
593 **RNA extraction and precipitation.** Briefly, following biofilm recovery after either 24 h, 48 h,
594 or 7 d incubation, 1 ml of the lung homogenate was added to 2 ml DNA LoBind tubes
595 (Eppendorf) alongside 500 µl sterile killing buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 20
596 mM NaN_3). The samples were then centrifuged for 1 min at 13,000 rpm, snap frozen, then

597 stored at -80 °C for a minimum of 1 h. Subsequently, samples were defrosted on ice and the
598 supernatant was carefully discarded without disturbing the pellet. The pellet was re-
599 suspended in 600 µl sterile LETS buffer (0.1 M LiCl, 0.01 M Na_2EDTA, 0.01 M Tris-HCl pH 7.5,
600 0.2% SDS) then transferred to 2 ml lysing matrix B tubes (MP Biomedicals) and bead beat in
601 a FastPrep-24 5G for three cycles: 6 m s^{-1} for 40 s then 5 min incubation on ice. All samples
602 were then incubated until they reached room temperature, centrifuged at 13,000 rpm for
603 10 min, then 600 µl 125:24:1 Phenol Chloroform Isoamyl alcohol (PCI) pH 4.5 (Invitrogen)
604 was added to each tube. The tubes were vortexed at ~14,000 rpm for 5 min, then
605 centrifuged for 5 min at 15,000 rpm at 4 °C. The top layer that had formed in each sample
606 was transferred to individual 2 ml RNase-free tubes (Sarstedt Ltd), and 1 volume of 125:24:1
607 PCI pH 4.5 was also added. The above 5 min vortex, centrifugation, and transfer steps were
608 repeated then a volume of 24:1 Chloroform Isoamyl alcohol (Sigma-Aldrich) was added. All
609 samples were centrifuged a further time for 5 min at 15,000 rpm, then the top layer was
610 transferred to individual 2 ml RNase-free tubes. A 0.1 volume of 3 M $NaCH_3COO$ pH 5.2 and
611 1 volume of isopropanol was added to each tube, the tubes were inverted, and stored at -20
612 °C overnight.
613
614 All samples were defrosted on ice and centrifuged for 15 min at 15,000 rpm, and the
615 supernatant was removed. The pellets were each re-suspended in 1 volume 70% (v/v)
616 ethanol then centrifuged at 4 °C for 15 min at 15,000 rpm, and the supernatant was
617 removed. The pellets were dried for ~15 min, re-suspended in 50 µl RNase-free water,
618 incubated on ice for 3 h, incubated at room temperature for 30 min, then snap frozen and
619 stored at – 80 °C.
620

621 **DNA removal.** To remove DNA, each sample was defrosted and 50 µl aliquots of maximum
622 concentration 200 µg ml^{-1} were transferred into PCR tubes. A 9:1 ratio of 10X DNase I buffer
623 (Invitrogen) was added to each aliquot, followed by 2 µl DNase I (Invitrogen), then the
624 samples were incubated for 30 min at 37 °C. Another 2 µl DNase I was added to each
625 reaction and the incubation was repeated. A volume of ethanol was added, then the
626 samples were transferred into Zymo-Spin ICC columns, and the Direct-zol RNA MiniPrep Plus
627 kit (Zymo Research) was followed as outlined previously [19]. DNA removal was confirmed
628 using 16S PCR and gel electrophoresis.
629
630 **RNA quality check and sequencing.** The final RNA concentration was determined using the
631 Qubit RNA broad range (BR) assay kit, and quality confirmed with the Agilent 2100
632 bioanalyzer system and RNA 6000 Pico Kit (Agilent). Depletion of any bacterial or
633 mammalian rRNA and Illumina library preparation for strand-specific RNA-sequencing were
634 then performed by Genewiz. Sequencing was performed on an Illumina Novaseq 150 bp
635 paired-end run.
636
637 **RNA-sequencing bioinformatic analyses.** All reads were quality checked using FastQC
638 v0.11.8 [47] initially and following every processing step. Read trimming was performed
639 using Trimmomatic v0.38 [48] and a minimum read length threshold of 25 bp [18]. Residual
640 eukaryotic and bacterial rRNA were filtered out of the reads using SortMeRNA v2.1b [49],
641 then the remaining reads were mapped to the pig genome (*Sus scrofa*: NCBI,
642 GCF_000003025.6) using HISAT2 v2.1.0 [50]. All reads that aligned were removed using
643 Seqtk v1.3-r106 [51], and the remaining reads were aligned to the *P. aeruginosa* UCBPP-
644 PA14 genome (NCBI, GCF_000014625.1) using BWA v0.7.17-r1188 aligner with the MEM

645 algorithm [52]. The reads mapped to coding sequences were counted using the
646 "featureCounts", part of the Rsubread v2.0.1 R package [53], against the *P. aeruginosa*
647 UCBPP-PA14 annotation from pseudomonas.com [3]. Count data was normalised using the
648 rlog transformation function, part of the R package DESeq2 v1.26.0 [54]. Principal
649 component analysis (PCA) was then performed using the "plotPCA" function within DESeq2,
650 using genes (n = 5,829), and 95% confidence ellipses added using ggpubr v0.4.0 [55].
651
652 Differential expression analysis comparing the three time points: 24 h, 48 h, and 7 d, was
653 performed using the R package DESeq2 [54]. The threshold for significant differential
654 expression was an adjusted *P* value < 0.05 (Benjamini-Hochberg procedure to control the
655 false discovery rate) and log2 fold change ≥ |1.5|. A Venn diagram of the numbers of
656 differentially expressed genes was produced using VennDiagram v1.7.0 R package [56].
657 KEGG pathway enrichment analysis was then performed using the differentially expressed
658 genes using the "enrichKEGG" function from the clusterProfiler v3.14.3 R package [57] and
659 the organism KEGG code "pau" for *P. aeruginosa* UCBPP-PA14. A KEGG pathway was
660 considered significantly enriched if the adjusted *P* value < 0.05 (Benjamini-Hochberg
661 procedure). The antimicrobial resistance genes studied were sourced from
662 pseudomonas.com [3], using the Comprehensive Antibiotic Resistance Database (CARD)
663 predictions [58]. Genes associated with the specific pathways: quorum sensing and biofilm
664 formation, were sourced from the KEGG database [59-61].
665
666 **Scanning electron microscopy.** Following EVPL dissection, infection with *P. aeruginosa*
667 PA14, and incubation at 37 °C for either 24 h, 48 h, or 7 d, lung tissue samples were fixed in

668 0.5 ml 2.5% glutaraldehyde solution in PBS, in a 24-well plate at 4 °C overnight. Uninfected
669 tissue was also prepared as a negative control for *P. aeruginosa* infection.
670
671 Subsequently the 2.5% glutaraldehyde solution was removed, and the tissue samples were
672 rinsed with PBS three times. Each tissue piece was then transferred to individual wells of a
673 new 24-well plate and passed through an ethanol gradient at the following concentrations
674 (v/v): 20%, 50%, 70%, 90%, 100%, and a further 100%, each for 1 h. The samples were
675 transferred to individual wells of a further 24-well plate, and 0.5 ml hexamethyldisilazane
676 (Electronic grade, 99+%) (HDMS) (VWR International Ltd) was added. The plate was
677 incubated at room temperature for 3 h, then the HDMS solution was discarded. The tissue
678 pieces were transferred to a new 24-well plate and left to dry in a laminar flow cabinet for 1
679 h. Double-sided copper tape (Agar scientific) was added to the scanning electron microscopy
680 sample holders (Agar scientific), then the tissue samples were placed on top. All samples
681 were then sputtered with carbon by using a carbon coater (SC7620 Mini Sputter
682 Coater/Glow Discharge System) and immediately imaged using a Zeiss Gemini scanning
683 electron microscope with an InLens detector, at a voltage of 1 kV. The data was analysed
684 using Omero5.6 [30].
685
686 **Data presentation and statistical analyses.** All bar graphs were produced using DataGraph
687 v4.7.1 [62] on Mac OS Mojave v10.14.6. Analysis of variance (ANOVA) was performed on
688 antibiotic treatment CFU data and post-hoc Tukey HSD tests. All statistical analyses including
689 ANOVA and differential expression analysis were performed using RStudio v1.2.1335
690 (www.rstudio.com) on Mac OS Mojave v10.14.6.
691

692 **Data availability.** A full list of the significant DEGs from the three contrasts reported are
693 provided as a data supplement. The raw sequence data is available at the European
694 Nucleotide Archive (accession number: PRJEB48552).
695

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697

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709

710 ## Author Contributions

711

712 N.E.H. and F.H. designed research; N.E.H., F.A., and R.G.M. performed research; N.E.H. and
713 F.A. analyzed data; N.E.H. and F.A. wrote the paper; N.E.H., F.A., R.G.M., and F.H. edited the
714 paper.

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716 ## Competing Interests

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718 The authors declare no conflict of interest.

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