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Research Article

Development of an *ex vivo* porcine lung model for studying growth, virulence and signalling of *Pseudomonas aeruginosa*

Running title: *P. aeruginosa* infection of *ex vivo* pig lung

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24 **Abstract**

25

26 Research into chronic infection by bacterial pathogens such as *Pseudomonas aeruginosa* uses various
27 *in vitro* and live host models. While these have increased our understanding of pathogen growth,
28 virulence and evolution, each model has certain limitations. *In vitro* models cannot recapitulate the
29 complex spatial structure of host organs, while experiments on live hosts are limited in terms of sample
30 size and infection duration for ethical reasons; live mammal models also require specialized facilities
31 which are costly to run. To address this, we have developed an *ex vivo* pig lung (EVPL) model for
32 quantifying *Pseudomonas aeruginosa* growth, quorum sensing (QS), virulence factor production and
33 tissue damage in an environment that mimics a chronically-infected cystic fibrosis (CF) lung. In a first
34 test of our model, we show that *lasR* mutants, which do not respond to 3-oxo-C12-HSL-mediated QS,
35 exhibit reduced virulence factor production in EVPL. We also show that *lasR* mutants grow as well as,
36 or better than a corresponding wild type strain in EVPL. *lasR* mutants frequently and repeatedly arise
37 during chronic CF lung infection, but the evolutionary forces governing their appearance and spread
38 are not clear. Our data are not consistent with the hypothesis that *lasR* mutants act as social ‘cheats’ in
39 the lung; rather, our results support the hypothesis that *lasR* mutants are more adapted to the lung
40 environment. More generally, this model will facilitate improved studies of microbial disease,
41 especially studies of how cells of the same and different species interact in polymicrobial infections in
42 a spatially structured environment.

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47 **Introduction**

48 *Pseudomonas aeruginosa* commonly causes nosocomial infections and is a particular danger for people
49 with cystic fibrosis (CF), in whom it establishes chronic lung infections (1). These are virtually
50 impossible to clear with current therapeutic regimens, due to ciliary malfunction, the build-up of
51 adhesive mucus in the CF airways (2), antibiotic resistance and *P. aeruginosa*'s ability to produce
52 protective polysaccharide capsules (3). People with CF experience decades of chronic infection with
53 repeated episodes of acute pulmonary exacerbation (1). During this time, *P. aeruginosa* evolves and
54 diversifies; mutants with altered production of virulence factors are commonly isolated from patients
55 (4-11), as are mutants that are impaired in quorum sensing (QS) (9, 10, 12).

56

57 Our understanding of the evolutionary pressures on *P. aeruginosa* during chronic lung infection, and
58 how these may be mediated by population or wider microbial community structure in these spatially-
59 structured organs, is currently limited (13-15). Yet, the evolutionary ecology of lung infections is likely
60 a key factor in morbidity and response to clinical interventions (13, 16).

61

62 To clarify the role played by different *P. aeruginosa* virulence factors and by inter-microbial
63 interactions in chronic infection, we need model systems that closely mimic a lung environment, but
64 are tractable in the lab and amenable to high-throughput experiments. A variety of *in vitro* growth
65 conditions and insect or rodent hosts have been used to study *P. aeruginosa* populations. The pros and
66 cons of these systems are outlined in Table 1, along with those of an under-used model host: *ex vivo*
67 sections of porcine lung. This model is useful for several reasons. First, pigs are arguably better models
68 for studying human disease than are rodents or invertebrates (17-19). Second, lungs can be obtained
69 from butchers: as little or no lung tissue is used in food production, lungs are a) cheap and b) a waste
70 product whose use does not raise ethical questions about the slaughter of animals for research. Third,
71 many small sections of tissue can be kept in culture for several weeks (20, 21). Finally, and crucially,
72 the spatial structure of the tissue is retained and microbes can be visualized within the tissue by
73 conventional or confocal microscopy. Histopathological changes can also be examined.

74

75 We developed this model for quantitative studies of *P. aeruginosa* growth and exoproduct production.
76 We focussed on the well characterized PAO1 wild type (WT) strain and two *lasR* mutant strains which
77 do not respond to the QS signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (49).

78 QS controls the expression of various exoproducts (49) and facilitates the establishment of acute
79 infection (31-36). However, mutants that have lost LasR function, and so do not respond to 3-oxo-C12-
80 HSL, commonly arise in chronic CF infections (9, 10, 12) and ventilator-associated pneumonia (50).
81 There is debate over whether *lasR* mutants are social ‘cheats’ that benefit from the presence of WT
82 cells (51, 52), or whether they are adapted to the chronic lung environment. Resolving this question is
83 important because it will affect the likely clinical success of QS inhibitors, which have been suggested
84 as novel anti-virulence agents and antibiotic adjuvants (53, 54). We therefore compared the growth of
85 *lasR* mutants with that of the WT in single-genotype and mixed infections in *ex vivo* pig lungs (EVPL).
86 We also measured the production of 3-oxo-C12-HSL and of two groups of virulence factors whose
87 expression is regulated by QS and that are linked with virulence in acute infection or with acute
88 exacerbation and declining lung function in people with CF: tissue-degrading proteases (55) and redox-
89 active phenazines (55-57). We also assayed for the siderophores pyoverdine and pyochelin, as these
90 have been shown to be necessary for acute infection (27, 58) and their role in chronic infection has
91 been much discussed (59-62).

92

93 We report three key results: 1) we can detect differential production of 3-oxo-C12-HSL, protease and
94 phenazine compounds by WT and QS mutant *P. aeruginosa* colonising EVPL; 2) consistent with this,
95 *lasR* mutants cause less pathological change to the host tissue; and 3) *lasR* mutants grow as well as or
96 better than the WT in EVPL in single infections, and a marked *lasR* mutant had equal fitness to the WT
97 in a mixed infection. Therefore, in this context, *lasR* mutants do not behave as social cheats: rather,
98 they grow well in this chronic infection model.

99

100 **Materials and Methods**

101

102 **Bacterial strains and culture conditions**

103 The Nottingham PAO1 strain of *P. aeruginosa* was used as the wild type (WT). A PAO1 mutant,
104 carrying a gentamicin resistance cassette inserted into the *lasR* gene, was used as a marked *lasR*-null
105 mutant (*lasR*::Gm, (26). For comparison, an unmarked PAO1 clone with a clean deletion of *lasR*
106 (Δ *lasR*) was also used. Preliminary work suggested that levels of phenazines produced in our infection
107 model were too low to be assayed via spectrophotometry, so we used PAO1 WT and PAO1 Δ *lasR*
108 strains carrying a reporter construct for one of the main phenazine biosynthetic operons (*phzA1*::*lux*
109 fusion; Higgins et al., manuscript in preparation). Infected cubes of lung tissue were cultured in

110 Artificial Sputum Medium (ASM: (24)) for 24 hours at 37°C on an orbital shaker. ASM mimics the
111 chemical composition of CF sputum but is not viscous. All media used were supplemented with
112 50µg/ml ampicillin to minimize the growth of any resident bacteria present in the lung cubes.

113

114 **Preliminary work and observations**

115 Lungs were purchased from a butcher (A Holmes and Son, Coalville, Leicestershire). We conducted
116 preliminary work on five lungs and determined that the tissue was healthy and not damaged by the
117 process of dissecting and preparing tissue (Figure S1). We used the work of Nunes et al. (42) as a
118 starting point to develop a protocol for dissecting out relatively regular cubes of alveolar tissue of
119 approx. 5mm³ (125µl), inoculating with c.10⁴-10⁵ *P. aeruginosa* cells and culturing the infected cubes
120 in ASM for up to seven days. Finally, we determined that we could a) visualize luminescent reporter
121 bacteria in the cubes; b) homogenize infected tissue to recover live bacteria; and c) conduct quantitative
122 assays on lung homogenate for the presence of 3-oxo-C12 HSL, total protease, pyocyanin and light
123 production by luminescent (*lux*) reporters. We also verified that resident lung bacteria were present at
124 very low levels, being almost entirely outcompeted by *P. aeruginosa* in infected tissue.

125

126 **Ethics statement**

127 All lung material was purchased from a high-street butcher and was sourced from animals already
128 slaughtered for meat; ethical approval was therefore not required for this study.

129

130 **Preparation of lung material**

131 The final protocol for the preparation, inoculation and culture of EVPL is shown in Figure 1. Cubes of
132 approx. 5mm³ were dissected from the ventral surface of the left caudal lobe of three sets of lungs
133 using a sterile mounted razor blade. Large bronchioles and veins were avoided in order to keep the
134 cubes as comparable as possible. Prior to dissection, the ventral surface of the pleura was briefly (< 1
135 sec) seared with a hot pallet knife to kill surface contaminants from the abbatoir or butcher's shop. This
136 also rendered the pleura easier to cut. During dissection, the tissue to be used was washed three times
137 with cell culture medium (1:1 mix of RPMI-1640 and DMEM, Sigma Aldrich). The cubes were then
138 washed for a fourth time in ASM. Preliminary work confirmed that searing and washing did not cause
139 any visible damage to the pleura (light microscopy of formalin-fixed tissue) and that these processes
140 reduced the numbers of contaminating and/or resident bacterial cells present in the cubes. We aliquoted
141 400µl ASM supplemented with 0.8% agarose to individual wells of a sterile 24-well plate (to provide a

142 soft surface for the tissue to sit on) and placed cubes singly in wells on this surface. Cubes were
143 covered with 500µl liquid ASM. As a control experiment to explore the growth of the bacterial strains
144 in the absence of lung tissue, cultures were set up exactly as above, but in place of the lung cube an
145 extra 125µl liquid ASM was added. Three experimental replicates of this experiment were performed;
146 in each case five populations each of WT, *lasR::Gm*, Δ *lasR* and WT+*lasR::Gm* in a 1:1 mix were
147 inoculated.

148

149 **Inoculation of lung tissue**

150 Bacterial strains were grown overnight in Lysogeny Broth (LB), washed twice in phosphate-buffered
151 saline (PBS) and resuspended in ASM. Cubes were inoculated with c. 10^4 washed overnight culture
152 cells in 50µl ASM - or as a mock-infection control, with 50µl sterile ASM – using a 30G needle
153 attached to a disposable 1ml syringe. Cubes were then incubated for 24 hours at 37°C on an orbital
154 shaker. Figure S2 shows a schematic of the experiment.

155

156 **Assays**

157 After incubation, cubes were rinsed in 1ml PBS to remove loosely-adhering cells. Growth of bacteria
158 was assayed by homogenising cubes individually in 500µl phosphate-buffered saline with metal bead
159 tubes (Cambio) using a Precellys24 homogenizer, serially diluting the homogenate and plating aliquots
160 on LB plates to obtain single colonies. To score the relative frequencies of WT and *lasR* mutant cells in
161 mixed infections, aliquots were replica plated onto LB+20µg/ml gentamicin. In mixed infections, the
162 relative fitness of the mutant was calculated as follows:

163

$$v = \frac{x_2(1-x_1)}{x_1(1-x_2)}$$

164

165

166

167 where x_1 and x_2 are the initial and final frequencies of the mutant in the population, respectively. When
168 the two genotypes have equal fitness, $x_1 = x_2$ and $v = 1$. Values of $v < 1$ reflect the mutant being
169 outcompeted by the WT and values > 1 indicate that the mutant outcompetes the WT. To quantify QS
170 signals, total protease, pyocyanin and the siderophores pyoverdinin and pyochelin, an aliquot of the
171 homogenate was diluted tenfold in PBS and filtered using a 0.2 µm syringe-driven filter unit to remove
172 cells. This was stored at -20°C. The amount of the QS signal 3-oxo-C12-HSL in the diluted

173 homogenates was quantified using the pSB1075 *Escherichia coli* biosensor (63); briefly, 100 μ l of each
174 homogenate was mixed with 100 μ l of an overnight biosensor culture diluted to an OD₆₀₀ of ~0.1 and
175 the luminescence/OD₆₀₀ for each culture measured after 30 minutes of incubation at 37°C in a 96-well
176 plate. The assay was calibrated using purified 3-oxo-C12-HSL. To measure total protease, 100 μ l
177 homogenate was mixed with with 5 mg azocasein dissolved in 900 μ l 100 mM Tris-HCl + 1 mM CaCl₂
178 and the mixture was incubated with shaking for 2 hours at 37°C; the reaction was then stopped by
179 adding 100 μ l 120 mM EDTA and the absorbance of the supernatant read at 400 nm. This assay was
180 calibrated using known concentrations of purified proteinase K. Pyocyanin was quantified by
181 measuring absorbance of homogenates at 695 nm, pyoverdine by exciting with light at 400 nm and
182 measuring fluorescence at 460 nm (64) and pyochelin by exciting at 350 nm and measuring
183 fluorescence at 430 nm (65). To assay activity of the *phzA1* phenazine operon by reporter bacteria,
184 aliquots of non-filtered, undiluted homogenate were assayed for luminescence. Spectrophotometric
185 assays were carried out using either a Tecan Infinite F200 Pro (3-oxo-C12-HSL biosensor, protease,
186 luminescence) or a Molecular Devices SpectraMax M2 (pyoverdine, pyochelin, pyocyanin). Finally,
187 to assess tissue damage and bacterial growth, cubes were fixed in formalin, sectioned and stained with
188 haematoxylin and eosin (H&E) and Gram's stain and inspected under a light microscope (Nikon
189 Eclipse 50i with Digital Sight DS-U3 camera).

190

191 **Statistical analysis**

192 ANOVA with Type II sums of squares (*car* package (66) in R 2.14.0 (67)) was used to test for main
193 effects of lung and inoculum (sterile ASM and/or the different bacterial strains) and for differential
194 effect of inoculum in different lungs (lung \times inoculum interaction when the sterile ASM control was
195 included in the analysis, lung \times strain interaction when the sterile ASM control was excluded), on
196 dependent variables. Data on total number of colony-forming units (CFU), relative fitness of the
197 *lasR::Gm* mutant, 3-oxo-C12-HSL concentration, protease concentration and phenazine reporter
198 expression were transformed using the natural logarithm when they were used as dependent variables,
199 in order to meet the assumptions of ANOVA. All *p*-values are given for two-tailed tests.

200

201

202 **Results**

203

204 ***P. aeruginosa* causes visible tissue damage**

205 As shown in Figure S1, fresh uninfected lung cubes appeared healthy, with open alveoli surrounded by
206 thin, well-defined epithelium. To explore the effects of *lasR*-mediated QS on growth and virulence,
207 cubes were inoculated with c. 10^4 washed overnight culture cells of WT PAO1, two independent *lasR*
208 mutants (the insertional mutant PAO1 *lasR*::Gm and the clean knockout PAO1 Δ *lasR*), a mix of PAO1
209 + PAO1 *lasR*::Gm or a phenazine bioreporter strain constructed in a WT or a Δ *lasR* background. As a
210 mock-infection control, cubes were inoculated with 50 μ l sterile Artificial Sputum Medium (ASM). A
211 visual overview of the final dissection and infection protocol is given in Figure 1 and a schematic of the
212 experimental design is given in Figure S2.

213

214 Mock-infected cubes retained their gross structural integrity over 24 hours at 37°C, while infected
215 cubes lost their shape and became soft, with visible green *P. aeruginosa* growth (Figure S3).
216 Microscopy of fixed and stained tissue sections showed that mock infected lungs showed minimal
217 histopathological changes when compared with lung tissue that was fixed and sectioned prior to
218 infection, with only small amounts of apoptotic/necrotic debris in the alveoli (Figure 2a,b); in most
219 cases, Gram staining did not show the presence of resident bacteria. Some sections of mock-infected
220 tissue exhibited areas of reduced alveolar volume reminiscent of areas of inflammation in living tissue
221 and the least histologically normal sample also contained large numbers of Gram-negative rods; it is
222 highly unlikely that these were *P. aeruginosa* as we never recovered *P. aeruginosa* when we plated out
223 mock-infected lung homogenate. As exemplified in Figure 2c,d, sections of tissue infected with the
224 PAO1 WT had no remaining alveolar structure and far fewer cell nuclei were visible than in mock-
225 infected tissue. Tissue preservation appeared slightly better in lung tissues infected with the *lasR*::Gm
226 mutant; as exemplified in Figure 2e,f, these were more reminiscent of highly inflamed tissue. In
227 infected tissues, Gram staining revealed large numbers of Gram-negative rods, which we presume to be
228 *P. aeruginosa* (Figure S4).

229

230 ***lasR* mutants do not show a growth disadvantage in EVPL**

231 We compared the fitness of WT and *lasR* mutant genotypes in single and mixed infections of EVPL. *P.*
232 *aeruginosa* grew in the lungs (Figure 3), reaching final densities of 6×10^5 - 4×10^9 CFU per cube (5×10^3
233 - 3×10^7 CFU per mm³ of tissue). The final density differed between strains ($F_{3,36} = 4.48$, $p = 0.009$) and
234 between lungs ($F_{2,36} = 19.2$, $p < 0.001$); crucially, the different strains showed consistent differences in
235 growth across the different lungs (interaction $F_{6,36} = 0.830$, $p = 0.555$). Post-hoc Tukey HSD

236 comparisons showed that QS mutants grew as well ($\Delta lasR$, $p = 0.297$) or better ($lasR::Gm$, $p = 0.049$)
237 than the WT in single infections. The mixed infections were initiated with a mixture comprising c. 60%
238 WT / 40% $lasR::Gm$ and these frequencies did not change over the incubation period: the relative
239 fitness of the mutant did not vary between lungs (ANOVA for effect of lung, $F_{2,7} = 0.04$, $p =$
240 0.97) and was not significantly different from 1 (post-hoc t -test, $t = 0.62$, $p = 0.56$).

241

242 To determine whether the relative fitness of the $lasR$ mutant was due to growth in lung tissue and not
243 simply to growth in ASM, we performed a control experiment in which the cube of EVPL was replaced
244 with a corresponding volume of ASM. Pure cultures of the $lasR::Gm$ and $\Delta lasR$ mutant grew to
245 approximately half the density of the WT ($p < 0.001$ and $p = 0.013$, respectively); the final density of
246 the mixed WT+ $lasR::Gm$ population did not differ significantly from that of the WT ($p = 0.060$). These
247 results are shown in Figure S5; p -values are from post-hoc tests after a fully-factorial ANOVA testing
248 for the effects of strain and experimental replicate (strain $F_{3,47} = 7.69$, $p < 0.001$). The $lasR::Gm$ mutant
249 was not able to take advantage of the WT in mixed culture; its relative fitness did not differ in pure and
250 mixed culture (fully factorial ANOVA including experimental replicate: (strain $F_{1,23} = 3.40$, $p = 0.078$)
251 and was significantly <1 in both cases (post-hoc t -test, $t = 2.25$, $p = 0.034$). This appeared to be due to
252 the WT growing better in the absence of EVPL than in its presence, but further work is needed to
253 explore this.

254

255

256 ***P. aeruginosa* virulence factor expression in EVPL is QS dependent**

257 We used an *E. coli* bioreporter (63) to measure 3-oxo-C12-HSL in cell-free homogenates of mock-
258 infected and infected lung. As shown in Figure 4 the amount of signal produced differed between
259 inocula ($F_{2,36} = 28.6$, $p < 0.001$) but not between lungs (main effect $F_{3,36} = 0.50$, $p = 0.611$; interaction
260 $F_{6,36} = 1.39$, $p = 0.24$). This was due to the WT infection producing more signal than the control or $lasR$
261 mutant infections (Tukey HSD tests $p < 0.001$; mutant infections did not differ from the control, $p >$
262 0.7). The WT produced, on average 16 nM 3-oxo-C12 HSL (range: 6-47 nM). We re-ran these analyses
263 excluding the mock-infected control and included total CFU in the cube as a covariate to eliminate the
264 possibility that any differences between strains were due to variability in population density; the results
265 were unchanged. Many secreted molecules that have been linked with virulence in acute infection
266 models, or with acute exacerbations or more rapid decline of lung function in CF, are under QS control.
267 These include tissue-degrading proteases (55) and redox-active phenazine compounds (55-57). We

268 then sought to determine whether mutations in *lasR* and concomitant loss of 3-oxo-C12-HSL led to
269 decreased production of protease, phenazines and siderophores in EVPL.

270

271 Consistent with lower levels of tissue damage (Figure 2), the *lasR* mutants produced less protease *per*
272 *capita* than the WT (strain $F_{3,36} = 8.77$, $p < 0.001$; lung $F_{2,36} = 12.5$, $p < 0.001$; interaction $F_{6,36} = 1.28$,
273 $p = 0.29$), and this translated into much lower total protease in lung cubes (Figure 4. Analysis including
274 mock-infected cubes: inoculum $F_{4,45} = 148$, $p < 0.001$; lung $F_{2,45} = 14.0$, $p < 0.001$; interaction $F_{8,45} =$
275 7.4 , $p < 0.001$). On average, the total protease activity in cubes infected with the $\Delta lasR$ mutant and the
276 *lasR::Gm* mutant was 16% and 12%, respectively, of that measured in WT-infected cubes. Mock-
277 infected cubes contained no measurable protease ($t = 0.581$, $p = 0.56$), underlining the loss of immune
278 activity in this model. We re-ran the *per capita* and total protease analyses excluding the mock-
279 infected control and included total CFU in the cube as a covariate to eliminate the possibility that our
280 results were due to variability in population density; the results for the main effects of lung and strain
281 were unchanged but the lungstrain interaction became non-significant in both cases ($p > 0.1$).

282

283 Similar results were obtained for the phenazine compound pyocyanin (Figure 6. Analysis including
284 mock-infected cubes: inoculum $F_{4,45} = 12.7$, $p < 0.001$; lung $F_{2,45} = 2.47$, $p = 0.096$; interaction $F_{8,36} =$
285 2.18 , $p = 0.047$; dropping two outliers from the control group did not affect these results). Visual
286 inspection of cubes infected with a luminescent reporter for the phenazine biosynthetic operon *phzAI*
287 using a photon-counting camera confirmed that this operon was expressed in infected cubes (Figure 7a)
288 and plating confirmed that phenazine reporter constructs grew to similar densities regardless of whether
289 they were in a WT or $\Delta lasR$ genetic background. A quantitative assay showed that per-CFU expression
290 of luminescence by the *phzAI* reporter construct was lower in the $\Delta lasR$ background than in the WT
291 (Figure 7b; $F_{1,12} = 37.9$, $p < 0.001$); on average, expression in the $\Delta lasR$ background was 45% of that
292 in the WT background, but the magnitude of this difference differed between lungs (main effect $F_{2,12} =$
293 1.22 , $p = 0.33$; interaction $F_{2,12} = 16.9$, $p < 0.001$). Again, including total CFU in the cubes as a
294 covariate did not affect the results for pyocyanin and *phzAI* reporter expression. We could not detect
295 the primary and secondary siderophores pyoverdine and pyochelin in lung homogenates using
296 excitation/emission assays, which have been shown to be sensitive to $\geq 10\mu\text{M}$ pyoverdine (FH,
297 unpublished data) and $\geq 2\mu\text{M}$ pyochelin (65).

298

299

300

301 **Discussion**

302

303 **Tractability and potential of EVPL as an infection model**

304 *Ex vivo* sections of pig lung are a tractable model for studying *P. aeruginosa* growth and virulence.
305 Mock-infected tissue was relatively histologically normal after 24 hours incubation in ASM at 37°C
306 and preliminary observations suggest that little further histological changes occur in mock-infected
307 tissue after a further six days incubation. *P. aeruginosa* cells could be visualized in EVPL using a light
308 microscope and readily recovered from tissue. Cell-free suspensions of homogenized tissue could be
309 assayed for a range of bacterial virulence factors. The relative growth of WT and *lasR* mutant bacteria
310 in EVPL contrasted with the situation in ASM alone: in this setting, the WT outgrew the mutants by a
311 factor of approximately 2:1, whereas in EVPL the mutants grew as well as, or slightly better than, the
312 WT.

313

314 A key advantage of EVPL is the chance to study bacterial virulence factor production, growth and cell-
315 cell interactions in a spatially structured environment. A diverse literature has explored the potential
316 effects on bacterial gene expression, growth and virulence of interactions between cells of the same or
317 different species (4, 16, 27, 68-73), and how population structure can affect these interactions (22, 23,
318 35, 74). However, while it is clear the CF infection community is spatially structured on a gross
319 anatomical level (15, 75, 76), we do not know whether this community is spatially ordered at a scale
320 relevant to bacterial cell-cell interactions. This means that it is hard to assess the likely efficacy of
321 proposed clinical interventions that rely on disrupting cell-cell interactions, such as QS inhibitors (54).
322 Further, spatial structuring of bacterial populations will affect other processes relevant to the
323 development of chronic infection, such as the dynamics of bacteriocin producing and sensitive strains
324 (77), plasmid transfer (78) and the evolution of antibiotic resistance (79, 80). The potential to
325 manipulate the infection community inoculated into EVPL, to study its evolution using conventional
326 and confocal microscopy of sections taken at various times post-inoculation, and to correlate aspects of
327 community diversity and structure with histopathology and levels of virulence factors, represents a
328 significant opportunity to study the extent and consequences of cell-cell interactions in lung tissue.

329

330 Clearly, EVPL also has limitations, and future work must identify these, circumvent them where
331 possible, and clarify the extent to which EVPL represents a chronically-infected human lung. Firstly,

332 we must acknowledge the high variance in the data presented in this manuscript. As we had no *a priori*
333 expectations of the likely level of variability or ease of replication, this should be mainly viewed as a
334 proof-of-principle study which can be built upon by ourselves and others. As a result of this work, we
335 now know that we can (a) readily access and process lungs in batches, and (b) easily cut several dozen
336 regular cubes of tissue from each lung. This knowledge, along with the ability to use the data presented
337 here in power calculations, will allow researchers to design larger-scale experiments which provide
338 more reliable estimates of between-strain or between-genotype differences. Secondly, if we are to
339 determine how well this model recapitulates chronic infection in humans, a detailed exploration of the
340 chemical environment in EVPL and how this co-evolves with infecting microbes over time (days –
341 weeks) is required. For instance, we do not yet know whether the reported chemical similarities
342 between human and pig lung are maintained in this *ex vivo* system, or whether the ASM needs to be
343 modified when it is used in conjunction with tissue (for example, ASM contains iron but if this is also
344 supplied in abundance by the lung tissue then overall levels of bioavailable iron may be unrealistically
345 high, and this could explain why siderophore gene expression appeared to be switched off in our
346 experiment). Further, the oxygen regime in infected lungs is likely to be an important factor, and
347 infection foci may become less aerobic over the course of infection (81, 82). Future work could
348 address how and when oxygen levels change inside sections of EVPL, and how this affects the growth
349 and virulence of *P. aeruginosa*. Finally, a careful comparison of results obtained in EVPL with those
350 obtained from live animal models, and with clinical data from CF patients where applicable, will help
351 us to determine whether the differences we observe between genotypes in this model are likely to be
352 meaningful *in vivo*.

353
354

355 **Role of QS and fitness consequences of *lasR* mutation in EVPL**

356 Levels of 3-oxo-C12-HSL accumulated to nanomolar levels after 24 hours' infection with WT PAO1.
357 It is hard to know how this corresponds to the level of expression in CF lungs, as studies using various
358 assay methods report concentrations ranging from femtomolar to micromolar in CF secretions and
359 tissues (83-88). We detected significant differences between WT PAO1 and *lasR* mutants, which do not
360 respond to 3-oxo-C12-HSL. *lasR* mutants produced no detectable 3-oxo-C12-HSL and significantly
361 less protease and pyocyanin than the WT; further, expression of one of the phenazine biosynthetic
362 operons, *phzA1*, was significantly reduced. Consistent with these results, *lasR* mutant infected tissue
363 exhibited qualitatively less tissue damage. We therefore conclude that *P. aeruginosa* 'senses a quorum'

364 in EVPL. Moreover, our results are consistent with reports that *P. aeruginosa* isolates from CF patients
 365 undergoing periods of acute exacerbation overproduce various QS-dependent exoproducts (8, 55, 89)
 366 and that, in *P. aeruginosa* mouse infection models, areas of tissue with higher AHL concentrations
 367 exhibit more severe pathological changes (86), and *lasR* mutants cause less tissue damage than the WT
 368 (36). That our *lasR* mutants showed reduced pyocyanin production is interesting, because in standard
 369 laboratory medium *in vitro*, *lasR* mutants have been reported to produce significantly more pyocyanin
 370 than the WT (90, 91). In contrast, other studies have shown that among *P. aeruginosa* clones isolated
 371 from CF patients, *lasR* mutation is often associated with a loss of pyocyanin production *in vitro* (55)
 372 (see also (92)).

373

374 A key finding from our study is that *lasR* mutants grew as well as or better than the WT in EVPL. This
 375 is noteworthy because there has been considerable debate about the evolutionary dynamics of *lasR*
 376 mutants in chronic infection. There are at least three possible explanations for the presence of *lasR* QS-
 377 blind mutants in chronic *P. aeruginosa* infections. First, loss of QS response could be adaptive,
 378 conferring a growth or persistence advantage in the context of an established infection. Second, *lasR*
 379 mutants may act as social ‘cheats’ and persist because they take advantage of co-infecting QS-
 380 proficient genotypes, whose QS-dependent exoproducts may benefit any cell in the vicinity, regardless
 381 of its own level of production (22, 93-96). Third, QS-blind mutants may be maladaptive, but arise due
 382 to recurrent mutation and persist at low frequencies due to stochastic evolutionary drift. It is difficult to
 383 choose which of these alternatives (if any) is correct, because there is very little quantitative data on the
 384 frequency of QS-blind mutants within chronically infected hosts and how this changes (or not) over
 385 time.

386

387 Generally, the first hypothesis – that QS-blind mutants have a fitness advantage – has had little support,
 388 because loss of *lasR* function reduces the ability of *P. aeruginosa* to establish acute infections (31-36).
 389 We also found that in ASM in the absence of pig lung, *lasR* mutants were less fit than the WT. These
 390 observations, combined with demonstrations that *lasR* mutants can act as WT exploiting cheats in some
 391 situations *in vitro* (22, 26, 97, 98) and in acute burn wound infections (32, 35) have led some
 392 researchers to give the second hypothesis serious consideration. This has led to the idea of deliberately
 393 introducing cheating mutants to trigger population collapse or to act as ‘Trojan horses’ for carrying
 394 useful alleles (e.g. antibiotic susceptibility) into infectious populations (e.g. (99)).

395

396 Our result is not consistent with the “social cheat” hypothesis. Rather, it adds weight to the first
397 hypothesis: that loss of LasR function enhances growth in chronic infection. The chemical environment
398 in chronically infected, damaged tissues may confer a growth advantage on *lasR*-null mutants;
399 D’Argenio et al (100) report that the relative growth of WT and *lasR*-null monocultures *in vitro* is
400 dependent on medium composition and that in some media, *lasR*-null mutants outgrow the WT. In
401 addition, Duan and Surette (101) show that changes to media composition can change the way that the
402 QS system reacts to cell density. Moreover, in one of the few studies to track the frequency of *lasR*-null
403 *P. aeruginosa* in human patients over the course of infection, Köhler et al (50) interpret their data as
404 showing that *lasR* mutants are cheats, but they report that patients colonized only by *lasR* mutants had
405 similar bacterial loads as those colonized only by the WT and this strongly suggests that these mutants
406 are not impaired in chronic persistence. For a detailed discussion of the evidence for adaptive loss of
407 LasR, we refer the reader to the review by Heurlier et al. (102), and for a detailed discussion of social
408 cheating to Ghoul et al (51).

409

410 We could not detect production of the siderophores pyoverdine and pyochelin in EVPL. While
411 siderophores are necessary for acute infections of mice (58) and waxmoth larvae (27), their role in
412 chronic CF lung infection is unclear because tissue damage and low oxygen levels may render iron
413 more accessible (103-106). Studies of CF sputum samples have shown that siderophores are
414 sometimes, but not always, present at detectable levels (61, 62).

415

416 **Future directions**

417 Further optimisation of EVPL could render it a realistic, ethical and high-throughput model for
418 studying the evolutionary ecology and pathology of chronic lung infection. As discussed above, several
419 questions over the biological realism of the model remain to be answered, but the methodology we
420 have developed for processing and handling lung tissue will allow us and others to address these in
421 detail. The model as presented here produced more extensive and less localized tissue damage than
422 seen in post-mortem CF lungs, but as we inoculated with a high dose of bacteria and used aerobic
423 culture conditions, this is not surprising. Now we have demonstrated the potential of EVPL, the next
424 steps in developing the model will be to find conditions that produce stable and long-lived infections
425 (e.g. by titrating number of cells inoculated, culturing under microaerobic or anaerobic conditions,
426 simulating key aspects of the host immune response). In this study, we used alveolar tissue to minimize
427 between-cube variation in structure, but future work could focus on dissecting regular sections of

428 bronchiole as these are the foci of infection in human CF lungs (107). In the future, EVPL could
429 especially enhance research into interactions between microbes in multi-species infections, which are
430 the norm in CF (13, 15, 108-110) and are increasingly recognized as important in other respiratory
431 diseases such as chronic obstructive pulmonary disease (COPD) and asthma (111-113).

432

433

434

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443

444 **Footnotes**

445 Author contributions: FH and SPD conceived and designed the study, FH and AM developed the
446 dissection and infection protocol, FH carried out experimental work and analysed the data, SH made
447 the *phzAI::lux* reporter and contributed to manuscript preparation, FH and SPD wrote the manuscript.

448

449 The authors declare no conflict of interest.

450

451 *This article contains supporting information online*

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454 **References**

455

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782

783 **Figure and Table Legends**

784

785 **Table 1.** Comparison of different model systems for studying pathogen social behaviour and virulence.

786

787 **Figure 1.** Schematic of the final protocol for preparation, infection and culture of EVPL.

788

789 **Figure 2.** Micrographs of tissue after 24 hours in ASM, fixed and stained with H&E which colors
790 nuclei dark blue and other structures (cytoplasm, collagen etc.) pink. a, b) mock infected control, c,d)
791 infected with WT *P. aeruginosa* and e,f) infected with the *lasR::Gm* mutant. Panels a), c) and e) show
792 tissue at 100X magnification with a 100 μ M scale bar; panels b), d) and f) show tissue at 400X with a
793 50 μ M scale bar. In panel a), note two bronchioles (Br) with diagnostic folded epithelium of brush
794 border, example of a blood vessel (V) and lace-like pattern of alveoli defined by thin epithelium
795 (example outlined, A). Small patches of cellular debris are visible in the alveoli (three examples
796 circled). In panel b), occasional cells with horseshoe-shaped nuclei (circled) are visible, which may
797 represent neutrophils, along with enucleate red blood cells (two examples boxed). Note in panels c) and
798 d) the loss of clear epithelium, lower number of nuclei and decreased volume of airspace. In panels e)
799 and f) this change is less extreme, with thickened outlines of epithelium still discernible.

800

801 **Figure 3.** Number of colony-forming units (CFU) of *P. aeruginosa* recovered from *ex vivo* pig lung
802 cubes after 24 hours of incubation in artificial sputum medium. Different symbols show cubes from
803 independent lungs and bars denote overall means. Where pairwise differences between strains were
804 found to be significant ($p < 0.05$) using Tukey HSD tests, this is indicated with an asterisk.

805

806 **Figure 4.** 3-oxo-C12-HSL signal in mock-inoculated and *P. aeruginosa* infected lung cubes after 24
807 hours incubation. Different symbols show cubes from independent lungs and bars denote overall
808 means. The amount of signal in the WT infected cubes was significantly greater than in cubes infected
809 with the other three strains (Tukey HSD tests, $p < 0.001$).

810

811 **Figure 5.** Total protease in mock-inoculated and *P. aeruginosa* infected lung cubes after 24 hours
812 incubation. Different symbols show cubes from independent lungs and bars denote overall means.

813 Where pairwise differences between strains were found to be significant ($p < 0.005$) using Tukey HSD
814 tests, this is indicated with an asterisk.

815

816 **Figure 6.** Pyocyanin (A_{695}) in mock-inoculated and *P. aeruginosa* infected lung cubes after 24 hours
817 incubation. Different symbols show cubes from independent lungs and bars denote overall means.

818 Where pairwise differences between strains were found to be significant ($p \leq 0.006$) using Tukey HSD
819 tests, this is indicated with an asterisk.

820

821 **Figure 7.** a) Photon-counting image of cubes taken from one lung after 24 hours incubation. Mock-
822 inoculated cubes and cubes infected with unlabeled NPAO1 show no luminescence, cubes infected
823 with *phzA1::lux* reporters show luminescence. b) Per-CFU expression of *phzA1* by *P. aeruginosa* in
824 lung cubes (arbitrary luminescence units divided by CFU and blanked on samples from cubes infected
825 with the unlabeled NPAO1). Different symbols show cubes from independent lungs and bars denote
826 overall means. Asterisk denotes significant difference between strains in ANOVA ($p < 0.001$).

827

828

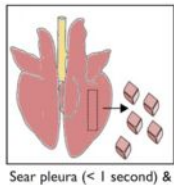
Table 1. Comparison of different model systems for studying pathogen social behaviour and virulence.

	Petri dish / test tube	Live invertebrate	Live mammal	Human cell or tissue culture	Ex vivo pig lung culture
Example studies	(22-26)	Waxworm (27,28), fruit fly (16) or nematode (29,30)	Usually mouse (14,31-36), occasionally other small mammals (37); also a CF pig model exists (38).	(39-41)	(20,21,42)
Chemical environment	Can be controlled to mimic CF sputum (24,25)	Not known	Mouse metabolome (17) and gene expression (19) very different from human	Controllable and can be made to mimic <i>in vivo</i> conditions	Metabolome more similar to human than a mouse is (17)
Spatial structure	Can be controlled, but artificial	Limited	Burn wounds: limited; lung infections: yes	Possible with scaffolding or organ sections	Very similar to human lung (18)
Immune system	None	Limited similarity with humans	Limited similarity with humans	Human	Very similar to human (18), but largely lost <i>ex vivo</i>
Infection timescale	Can study 100s-1,000s generations	Acute – host dies very quickly	At best semi-chronic. Rodent lung infections tend to be acute (days), though can sometimes last 1-4 weeks (43,44). Wound infections are usually limited to c. 3 weeks (K. Rumbaugh, pers. commun.)*	Days – weeks	Not known
Large sample sizes (tens +) possible?	Yes	Yes	No, due to cost and ethical considerations	Not usually	Yes
Cost	Low-medium	Low	High	Medium to set up, low to run	Low
Ease of method	Simple, requires only general microbiology techniques	Must learn how to inoculate, but otherwise simple	Requires specialised expertise, an animal licence and often a dedicated animal worker to carry out inoculation	Requires expertise and dedicated lab space/equipment to minimise risk of contaminating cell lines	Lungs are readily obtained from commercial butchers. We developed dissection, infection and culture techniques in c. 3 months
Ethical considerations	None	None	Yes – and limit sample size / infection duration	Minimal (donor informed consent must be obtained)	None if obtained from animals slaughtered for meat – little or no tissue is used for human consumption, so lungs are basically a waste product.
Review articles		(45,46)	(37,46,47)	(46,48)	

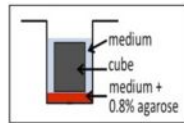
* Infection duration depends on local rules governing animal welfare, e.g. in the United Kingdom animals must be euthanased when the symptoms of infection become too severe.



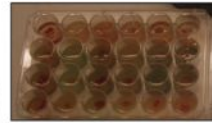
Lungs obtained from butcher (cut open to show healthy interior)



Sear pleura (< 1 second) & cut ~5mm cubes; wash tissue x3 with RPMI/DMEM and x1 with ASM during dissection



Transfer cubes to wells of 24-well plate as shown; inoculate (50µl) using hypodermic needle; cover with lid and incubate at 37°C on orbital shaker



After incubation (example cubes shown after 24 hours), wash cubes in 1ml PBS and perform required assays. Tissue may be homogenised by bead beating or fixed/stained for microscopy

