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1 # The social evolution of siderophore production in *Pseudomonas aeruginosa* is
2 # environmentally determined

3

4 *Running title: Environment and fitness of siderophore mutants*

5

6 Freya Harrison[1,2]
7 Alan McNally[3]
8 Ana C. da Silva[2]
9 Stephen P. Diggle[2]

10

11 [1] School of Life Sciences, Gibbet Hill Campus, University of Warwick, Coventry CV4 7AL, U.K.
12 [2] Centre for Biomolecular Sciences, School of Life Sciences, University Park, University of
13 Nottingham, Nottingham NG7 2RD, U. K.
14 [3] Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of
15 Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

16

17 *Authors for correspondence*
18 FH: f.harrison@warwick.ac.uk Tel. +44 (0)24 765 24744
19 SPD: steve.diggle@nottingham.ac.uk Tel.+44 (0)115 748 4267

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26

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28 The authors declare that there are no competing financial interests in relation to the work described.

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33 # Abstract

34 Bacteria secrete various exoproducts whose benefits can be shared by all cells in the vicinity. The potential
35 importance of these "public goods" in bacterial evolutionary ecology has been extensively studied. Cheating
36 by siderophore-null mutants of the opportunistic pathogen *Pseudomonas aeruginosa* has received particular
37 attention. The potential of siderophore mutants to attenuate virulence, and the possibility of exploiting this for
38 clinical ends, have generated a wealth of publications. However, the possibility that genotype · environment
39 interactions govern the evolutionary consequences of siderophore loss has been almost entirely ignored. A
40 review of the available literature revealed (i) widespread use of an undefined mutant as a siderophore cheat;
41 and (ii) a reliance on experiments conducted in iron-limited minimal medium. Whole genome sequencing of
42 the undefined mutant revealed a range of mutations affecting phenotypes other than siderophore production.
43 We then conducted cheating assays using defined deletion mutants, grown in conditions designed to model
44 infected fluids and tissue in CF lung infection and non-healing wounds. Depending on the environment, we
45 found that siderophore loss could lead to cheating, simple fitness defects, or no fitness effect at all. It is
46 therefore crucial to develop appropriate *in vitro* growth conditions in order to better predict the social evolution
47 of traits *in vivo*.
48

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49 **Introduction**

50

51 Bacteria are social organisms, displaying coordinated behaviours including quorum sensing (QS), biofilm
52 formation, and the production of shareable exoproducts (West et al 2007). Exoproducts which act as "public
53 goods" (including iron-scavenging siderophores, exoproteases, biofilm polymers, toxins and QS signals) are
54 vulnerable to "cheating": cells which do not produce a particular molecule avoid the costs of production but
55 reap the benefits of their neighbours' investment and increase their evolutionary fitness at the expense of
56 producers (Darch et al 2012, Diggle et al 2007, Griffin et al 2004, Harrison et al 2006, Jiricny et al 2010, Mund
57 et al in prep, Raymond et al 2012). The circumstances under which cooperation can be maintained in the
58 population over evolutionary time, versus those under which cheating prevails, have been predicted by theory
59 (Frank 1998, Hamilton 1964). Because bacteria are amenable to evolution experiments, they have been widely
60 used to test theoretical predictions (e.g. Diggle et al 2007, Griffin et al 2004, Kümmerli et al 2009a).
61 Siderophore production by the opportunistic pathogen *Pseudomonas aeruginosa* is a particularly tractable
62 workhorse for sociomicrobiology, facilitating tests of key evolutionary hypotheses (Table 1).

63

64 Early research into bacterial public goods focussed on using them as models to test general theory. But as the
65 field progressed, researchers began to suggest that public goods play important roles in infected hosts. This
66 led to suggestions that cooperation among pathogens could be manipulated for clinical ends, especially in hard-
67 to-treat chronic infections (e.g. Harrison et al 2006, Rumbaugh et al 2009, Brown et al 2009, Foster 2005). *P.*
68 *aeruginosa* causes various chronic infections in immunocompromised hosts, notably lung infections in people
69 with cystic fibrosis (CF) and wound infections in people with burns or diabetic ulcers (Friedrich et al 2015,
70 Hirsch and Tam 2010). Siderophore-null mutants can invade *in vitro* populations and cheat their way to high
71 density, resulting in population reduction. Researchers have hypothesised that the presence of siderophore
72 mutants in chronic infection isolates can be explained by cheating *in vivo*, and that siderophore mutants could
73 be used as "Trojan horses" to ferry antibiotic-susceptibility alleles into infections and thus render them curable
74 (Brown et al 2009, Harrison et al 2006).

75

76 One potential problem with this reasoning is the implicit assumption that gene expression and the fitness
77 consequences of mutations are comparable *in vitro* and *in vivo*. Technical advances have revealed just how
78 much bacterial transcriptomes (Croucher and Thomson 2010) and the fitness of loss-of-function mutants (Van
79 Opijnen et al 2009) vary across environments; indeed, environment may be a bigger predictor of *P. aeruginosa*
80 phenotype than its genetic background (Dötsch et al 2015). When the environment bacteria experience in a
81 specific infection context is carefully modelled in the lab, the importance of genotype × environment
82 interactions in determining fitness become very clear (Harrison et al 2014, Palmer et al 2007, Turner et al
83 2015).

84

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85 Typically, siderophore experiments are conducted in iron-limited minimal medium, creating clear costs and
86 benefits to siderophore production. While this may recapitulate the iron restriction encountered by pathogens
87 colonising healthy hosts during acute infection, chronic infections present an entirely different environment.
88 Tissue damage, a hyper-inflammatory response and disease-specific changes in host phenotype (e.g. increased
89 mucus volume and adhesivity in CF lungs, (Boucher 2007) are accompanied changes in the growth substrates
90 available to bacteria. In CF lungs, bacteria use amino acids released by damaged tissues, or from mucus, as
91 carbon sources (Flynn et al 2016, Palmer et al 2005, Palmer et al 2007), and iron is plentiful (Tyrrell and
92 Callaghan 2016). Consequently, bacterial gene expression – and presumably the roles played by virulence
93 factors – differ in chronic and acute contexts (Goodman et al 2004). Because lab models of the environments
94 encountered in CF lungs and soft-tissue wounds have been developed (Harrison et al 2014, Harrison and
95 Diggle in press, Palmer et al 2007, Turner et al 2015, Werthén et al 2010), there is a pressing need to re-assess
96 the role played by siderophore mutants in environments that better model chronic infection.
97
98 To assess and extend the potential of laboratory experiments to yield clinically useful data on the evolutionary
99 dynamics of siderophore production, we defined three aims. First, we reviewed published experimental work
100 on *P. aeruginosa* siderophore cooperation. This literature has not previously been systematically reviewed.
101 Our goal was to characterise any biases in the literature which could restrict its applicability to the various
102 environments in which this flexible opportunist can thrive. We found two such biases and defined two further
103 empirical aims to address these.
104
105 The first potential bias in the literature was that many experiments (by ourselves and others) used an
106 uncharacterised, UV-generated mutant (PAO9) as a siderophore cheat. We therefore conducted whole-genome
107 sequencing of this mutant to determine (i) the genetic basis of its siderophore-null phenotype, and (ii) whether
108 it carries other mutations that could affect the outcome of competition with the wild type. The second source
109 of bias was the lack of studies employing a well-defined model of chronic infection. We therefore determined
110 the fitness consequences of siderophore loss-of-function mutations in relatively well-characterised models of
111 CF lung infection and chronic soft-tissue wounds. Because we found many mutations that potentially affect
112 metabolism and growth in PAO9, we used siderophore deletion mutant of the siderophores pyoverdine and
113 pyochelin for these experiments. CF lung infection was modelled using (a) liquid artificial sputum medium
114 (ASM: Palmer et al 2007, Turner et al 2015); and (b) an *ex vivo* model of biofilm infection in which small
115 sections of porcine bronchiole are infected with bacteria and cultured in ASM (Harrison and Diggle in press).
116 Chronic wound infection was modelled using (a) synthetic wound fluid (SWF), and (b) synthetic wound fluid
117 solidified with collagen to form model soft-tissue plugs (Werthén et al 2010). We explicitly explored
118 differences between liquid medium and structured infection models because spatial structure affects the social
119 dynamics of public goods (Frank 1998, Kümmerli et al 2009c, Mund et al in prep), and because biofilm
120 formation in structured models could alter gene expression and the fitness consequences of mutation

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121 (Bjarnsholt et al 2013, Whiteley et al 2001). Our results demonstrate that whether siderophore mutants act as
122 cheats is dependent on the environment, and suggest that these mutants may not act as cheats *in vivo*.
123
124
125 **Meaterials & Methods**
126
127 *Bacterial strains*
128 *P. aeruginosa* ATCC 15692 (PAO1) was used as a wild type siderophore producer and clean Δ*pvdD* and
129 Δ*pvdD*Δ*pchEF* mutants in this background (Ghysels et al 2004) used as single pyoverdine and double
130 pyoverdine/pyochelin mutants, respectively. The UV-induced mutant PA6609 (PAO9) was derived from
131 PAO6049 (Hohnadel et al 1986); PAO6049 is a Tn5-induced methionine auxotroph derived from PAO1 (Rella
132 et al 1985).
133
134 *Collating and analysing experimental work on siderophore cheating*
135 To identify published experiments on siderophore cheating, we used Scopus.com to (i) conduct a literature
136 search and (ii) locate articles citing already-identified experimental and theoretical articles on siderophore
137 social evolution. For each experiment in each article, we recorded which strains/clones and media were used;
138 whether an explicit test for cooperation was conducted (relative fitness of mutant in pure/mixed culture with a
139 producer strain & growth or final density of pure/mixed cultures); the starting frequency of mutants in co-
140 culture experiments; the location of key data in the article; whether cheating was observed and any other key
141 conclusions. The diverse starting frequencies and mutants used make this data unsuitable for formal meta-
142 analysis, so we simply present the key characteristics and findings of the studies in a format that facilitates
143 qualitative comparison.
144
145 *Whole genome sequencing of PAO6609 (PAO9)*
146 PAO9 was cultured overnight in 10 ml Lysogeny Broth at 37°C on an orbital shaker. Genomic DNA was
147 extracted using a Sigma Aldrich GenElute Bacterial Genomic DNA Kit. Library preparation was performed
148 using the Nextera XT library preparation kit (Illumina), and 2 x 300 bp paired-end sequencing performed on
149 the Illumina MiSeq platform using a V3 sequencing cartridge, to approximately 50x coverage. Reads were
150 assembled using SPAdes run with the –careful flag, and annotated using Prokka. This produced an assembly
151 of 6,232,039 bp comprising 89 contigs with an N50 of 220,789. The assembly was compared with the reference
152 PAO1 genome (NCBI reference sequence NC_002516) using BLAST and the comparison visualised using
153 ACT to search for gene acquisition or loss events. SNP typing was performed by mapping the raw reads of
154 PAO9 against the PAO1 reference genome (NC_002516.2) using SMALT and Samtools. A total of 98.3% of
155 1.17M reads mapped to the reference, from which high fidelity SNPs were called using a cut off of minimum
156 allele frequency of 0.8, minimum quality score 30, and minimum depth of 8.
157

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158 *Growth conditions for cheating experiments*

159 We used five different growth environments. In each case, cultures were grown in 24-well microtitre plates
160 and incubated on a rocking platform at 37°C. (i) 2 ml casamino acids medium (CAA: 5 g casamino acids,
161 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, per litre) supplemented with 20 mM $NaHCO_3$ and
162 100 µg mL^{-1} human apo-transferrin (Sigma), cultured for 24 hours. (ii) 2 ml artificial sputum medium (ASM)
163 following recipe in Palmer et al (2007), cultured for 24 or 48 hours. (iii) 5 mm^2 pig bronchiole + 400 µl ASM
164 following protocol in Harrison and Diggle (in press), cultured for 96 hours. (iv) 2 ml synthetic wound fluid
165 (50% v/v peptone water / fetal bovine serum following Werthén et al (2010)), cultured for 24 or 48 hours. (v)
166 400 µl synthetic chronic wound (SCW: SWF solidified with rat tail collagen following protocol in Werthén et
167 al, (2010)), cultured for 48 hours. For environments ii-v, we measured wild-type siderophore production and
168 calculated the fitness of siderophore mutants relative to the wild type in clonal and mixed culture. Four or five
169 replica cultures were inoculated with (a) wild-type, (b) Δ*pvdD* (c) Δ*pvdD*Δ*pchEF*, (d) 50% wild-type + 50%
170 Δ*pvdD* or (e) 50% wild-type + 50% Δ*pvdD*Δ*pchEF* bacteria for each environment/culture time combination,
171 and each experiments was repeated twice to yield two experimental blocks. For environment i (CAA), we
172 simply measured siderophore production by four replica populations of the wild type for comparison with
173 environments ii-v.

174 The density of the inoculum was kept as consistent as possible across environments and population
175 types, as cell density can affect the outcome of wild-type – siderophore-null co-culture experiments (Ross-
176 Gillespie et al 2009). For experiments in CAA, ASM and SWF, each culture was inoculated with a total of two
177 colonies of the relevant genotype(s), picked from LB plates using a 200 µl pipette tip. For experiments in *ex*
178 *vivo* bronchiole and SCW, each culture was inoculated with two colonies of the relevant genotype(s), picked
179 from LB plates using an insulin syringe fitted with a 30 G needle.

180 To construct time courses of bacterial growth in ASM, two colonies of (a) wild-type, (b) Δ*pvdD* or
181 (c) Δ*pvdD*Δ*pchEF* were inoculated into four 2 ml aliquots of ASM in a 24-well microtitre plate. This was
182 incubated at 37°C in a Tecan Infinite 200 Pro multimode reader for 18 hours; every 20 minutes, the plate was
183 shaken for two seconds and absorbance of each well read at 400 nm. (Reading at more traditional values of
184 600-700 nm risks interference from absorbance by pyoverdine in wild-type cultures).

185

186 *Statistical analyses*

187 Data were analysed in R 3.3.0 (Team 2016) using general linear models and ANOVA. To meet model
188 assumptions of homoscedasticity and normality of residuals, fitness data from SWF were log-transformed and
189 fitness data from ASM and *ex vivo* bronchiole square-root transformed; fitness data from SCWs did not require
190 transformation. Where missing values caused non-orthogonality, the *car* package (Fox and Weisberg 2011)
191 was used to perform ANOVA with Type II sums of squares, returning correct F-ratios for main effects in
192 models containing an interaction. Fitted means and confidence intervals were retrieved, and post-hoc contrasts
193 tested, using the *lsmeans* package (Lenth 2013). Post-hoc multiple comparisons against a control were
194 implemented using Dunnett's test in the *multcomp* package (Hothorn et al 2008).

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195
196 *Data availability*
197 Raw sequence data has been deposited in the European Nucleotide Archive (Accession number:
198 ERR1725797). Summary information on mutations in PAO9 (Fig 1) and raw data for experiments depicted in
199 Figures 2-5 and S2 are available as Supplementary Information at the *Nature Ecology & Evolution* website.
200
201 **Results**
202
203 **Defining key research priorities: analysis of published experiments.**
204 A review of published experiments on *P. aeruginosa* siderophore mutants and cheating identified 33
205 experiments in 18 published articles, summarised in Table 1. Two-thirds of the experiments used the well-
206 characterised lab strain PAO1 as the siderophore-producing wild-type, and 5 experiments used clinical or
207 environmental isolates. Of the 28 experiments that did not focus on natural isolates, 14 used defined
208 siderophore mutants. These included deletions of the *pvdD/pvdF* and/or *pchEF* loci, which are involved in the
209 biosynthesis of the siderophores pyoverdine and the pyochelin, respectively. Of the remainder, 9 used the UV-
210 induced mutant PAO9 and 5 used spontaneous or evolved mutants.
211
212 31/33 experiments in Table 1 were conducted in CAA minimal medium, in all but one case made further iron-
213 limited by the addition of the human iron chelator transferrin. This medium was not designed to reflect any
214 specific natural environment: it was optimised to ensure that siderophores were necessary for growth, and
215 acted as a public good, when bacteria were cultured in it (Griffin et al 2004). This makes it ideal for
216 experiments to test evolutionary theory about why and how different social strategies can be selected for.
217 Experiments in iron-limited CAA have, for instance, revealed that the potential for siderophore mutants to
218 cheat can be curtailed by the scale of competition in a metapopulation (Griffin et al 2004) or by growth in a
219 structured environment, where spatial segregation prevents mutants from accessing siderophores produced by
220 wild types (Kümmerli et al, 2009c). As an opportunist with a large, flexible metabolome and secretome, *P.*
221 *aeruginosa* can persist in a range of environments. Some of these may be approximated by iron-limited
222 minimal media. For instance, experiments conducted in CAA may therefore be useful for understanding the
223 dynamics of siderophore genotypes/phenotypes at the onset of acute infection in healthy host tissues, where
224 iron is sequestered by high-affinity host chelators (including transferrin), or in nutrient-poor abiotic
225 environments.
226
227 One study explicitly tested the effect of environmental variables on siderophore production in monoculture.
228 Dumas et al (2013) dissected the relative roles of pyoverdine and pyochelin, suggesting that while pyoverdine
229 is the primary siderophore expressed under severe iron limitation, under moderate iron limitation pyochelin
230 production dominates. The authors used parameters estimated from these experiments to simulate wild-type –
231 siderophore mutant competition in a range of environmental scenarios, and found that the fitness consequences

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of siderophore-null mutations varied. Based on these results, the authors suggested that pyoverdine-deficient mutants may be favoured in chronic infection due to better growth in high-iron, low-pH environments, rather than because they are cheats. The importance of environmental iron regime and pyoverdine/pyochelin switching in determining the fitness consequences of siderophore mutants has also been explored in a suite of experiments using the closely-related bacterium *P. fluorescens*: pyoverdine mutants of this species were shown to cheat under severe, but not moderate, iron limitation (Zhang and Rainey 2013, Kümmerli and Ross-Gillespie 2013).

Further, the strength of siderophore cheating may be constrained. Cheating could become self-limiting as increases in mutant frequency alter the cost:benefit ratio of losing siderophore production, or the cost:benefit ratio may be different in actively-growing versus established populations. In many studies that explored a range of starting frequencies, mutants acts cheats only when they are initially inoculated at frequencies ≤ 0.1, which raises questions about the ability of these mutants to retain cheat at, and persist at, higher frequencies. In some cases, even though co-culture increased mutant relative fitness to > 1, there was no detrimental effect on total population density. These observations call into question the ability of siderophore mutants to act as "Trojan horses" to treat infection, as does the observation in one study (Ghoul et al 2016) that a siderophore mutant only cheats if added to wild-type cultures before stationary phase.

Therefore, whether siderophore-null mutants act as cheats in chronic infection remains an open question. It is possible that increased availability of iron in chronically-damaged tissues means that the benefits of siderophore production are reduced and that production is downregulated, removing any advantage to siderophore-null genotypes. Alternatively, siderophores may be beneficial but not exploitable by potential cheats: this could result from spatial structuring, persistence in stationary phase, and/or a reliance on siderophore recycling rather than continued production (Kümmerli & Brown 2010).

Whole genome sequence of a commonly-used siderophore cheat, PAO9

The nature of the mutation leading to the siderophore-negative phenotype in PAO9 is not known. Further, as UV mutagenesis is non-specific, it is likely that this strain carries additional mutations that influence other important aspects of its phenotype, or moderate the fitness consequences siderophore loss. Without knowing the exact genotype of PAO9, we cannot be sure that empirical results obtained using it are the result of its siderophore phenotype alone. We performed whole genome sequencing of this strain and mapped the raw sequence data against a PAO1 reference genome (NCBI Reference Sequence: NC_002516.2, Stover et al 2000). The results are summarised in Figure 1. PAO9 harbours a single ~6.6kb deletion, removing most of the non-ribosomal peptide synthetase locus *pvdJ*, which is involved in synthesising the pyoverdine side chain. This deletion also removes part of the open reading frame immediately upstream of *pvdJ*, PA2402. PA2402 encodes a probable non-ribosomal peptide synthetase of unknown function. We also found 90 high-resolution SNPs. The details of the deletion and SNPS are provided as Supplementary Information. Briefly, none of the

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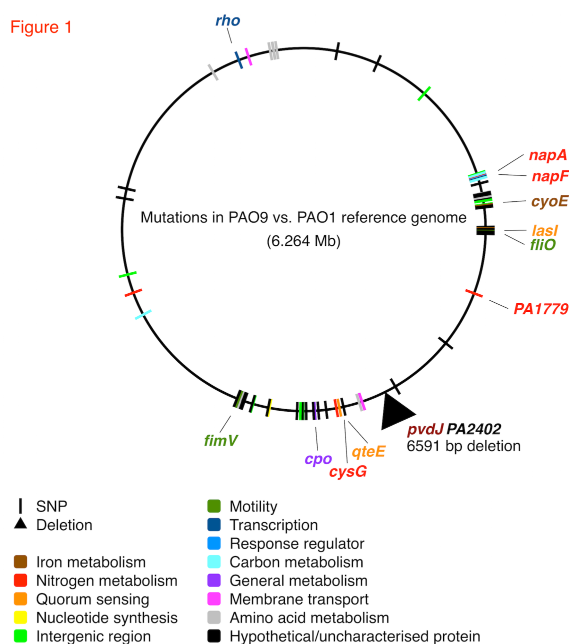
269 SNPs were in loci associated with pyoverdine or pyochelin biosynthesis. However, we found SNPs resulting
270 in mis-sense mutations in *cyoE*, which is involved in iron metabolism, in four loci associated with nitrogen
271 metabolism (*napA*, *napF*, *cysG*, PA1779), two loci involved in quorum sensing (*lasI*, *qteE*), two loci involved
272 in motility (*fliO*, *fimV*) and the *cpo* gene, which is involved in the biosynthesis of many organochlorine
273 compounds: all of these mutations could potentially affect growth and/or virulence. Finally, we found a mis-
274 sense mutation in the *rho* transcription termination factor. Further work would be needed to deduce the effects
275 of this mutation, but the possibility of generalised defects in the control of gene expression cannot be ruled
276 out. In summary, PAO9 harbours numerous mutations in addition to the *pvdJ* partial deletion that likely make
277 it an unreliable strain to use for empirical studies of the fitness and virulence consequences of siderophore loss.
278
279
280



281
282
283 **Figure 1.** Location of one deletion and 90 SNPs in the genome of PAO6609 (PAO9), mapped against a PAO1
284 reference sequence (NC_002516.2). SNPs are colour coded by functional class of the locus affected. 11 SNPs
285 that that result in amino acid substitution and which seem most likely to affect growth and virulence are
286 highlighted and the gene name given.
287
288

289 **Production of siderophores by *P. aeruginosa* in models of chronic lung and wound infections.**

290 Because of the problems identified with PAO9, we used defined siderophore mutants to test the potential for
291 social cheating in laboratory conditions that have been developed to represent specific chronic infection
292 contexts, with strict attention to maximising likely ecological validity. As discussed above, while most research
293 has focused on pyoverdine, under less severe iron limitation the metabolically cheaper siderophore pyochelin

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294 may take on a primary role in iron chelation (Dumas et al 2013, Kümmerli and Ross-Gillespie 2013). We
295 therefore used a standard wild-type lab strain (PAO1) and two isogenic deletion mutants: a single pyoverdine
296 knock-out (Δ*pvdD*) and a double pyoverdine/pyochelin knockout (Δ*pvdD*Δ*pchEF*) constructed using allelic
297 exchange (Ghysels et al 2004). Both mutants have been reported to act as social cheats in iron-limited CAA,
298 when inoculated in co-culture with the wild type at starting frequencies of up to 50% (Table 1, (Kümmerli et
299 al 2009b, 2015; Kümmerli and Brown 2010).
300
301 We first verified that PAO1 produces siderophores in our chronic infection models, and compared production
302 levels with those in iron-limited CAA. Detectable levels of pyoverdine and pyochelin were produced in all
303 environments. Per-cell levels of pyoverdine and pyochelin were (i) positively correlated and (ii) higher in lung
304 and wound infection models than in liquid ASM or SWF (despite lower cells densities in infection models than
305 in corresponding liquid medium) (Figure S1). Consistent with the suggestion of Dumas et al (2013), the
306 pyochelin:pyoverdine ratio was generally higher in chronic infection models and media than in CAA. Replica
307 cultures in *ex vivo* lung and synthetic wounds were more variable than replica cultures in liquid media.
308
309 **Social dynamics of siderophore mutants in artificial CF sputum are influenced by genotype and culture**
310 **time.**
311 Mutant and wild-type bacteria were grown in pure culture and each mutant was grown in mixed culture with
312 the wild type, with a starting frequency of 50%, in ASM. After 24 and 48 hours of growth, total population
313 densities were determined and the relative fitness of each mutant in pure and mixed culture calculated. As
314 shown Figure 2a, neither mutant had a relative fitness significantly different from 1 when grown in pure
315 culture. Δ*pvdD* mutant fitness was unaffected by the wild type, but the Δ*pvdD*Δ*pchEF* mutant was
316 outcompeted in mixed culture. These results are not consistent with either mutant acting as a cheat: they do
317 not show a disadvantage when cultured alone, and do not benefit from growth with the wild type.
318
319 After 48 hours (Figure 2b), the results were different. The Δ*pvdD* mutant now showed cheating dynamics: it
320 was less fit than the wild type when grown in pure culture but as fit as the wild type in mixed culture. As
321 predicted for a textbook "cheat," the presence of the Δ*pvdD* mutant reduced total population density: mixed
322 cultures reached similar densities to pure Δ*pvdD* cultures (Figure 2c). The Δ*pvdD*Δ*pchEF* mutant was simply
323 less fit than the wild type regardless of culture condition.
324
325 Time course experiments (Figure S2) suggested that the Δ*pvdD* mutant is disadvantaged in pure culture in
326 ASM because it ceases logarithmic growth earlier than the wild type, reaching a lower yield in stationary
327 phase. The Δ*pvdD*Δ*pchEF* mutant has the double disadvantage of a longer lag phase and an earlier cessation
328 of logarithmic growth. This is consistent with the differing fitness of the mutants at 24 vs. 48 hours: continued
329 growth and siderophore production by the wild type from 24-48 h presumably provide an opportunity for the
330 Δ*pvdD* mutant to cheat.

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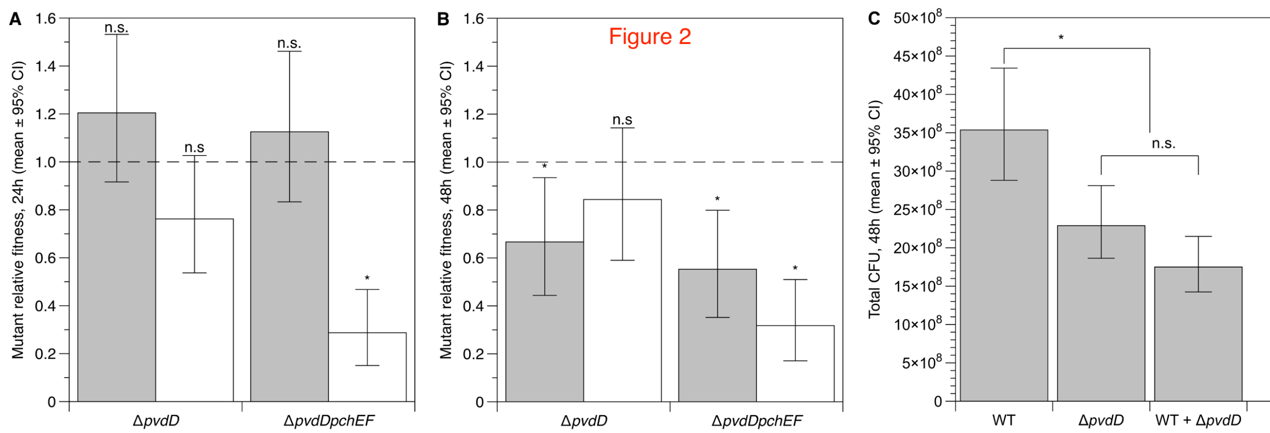


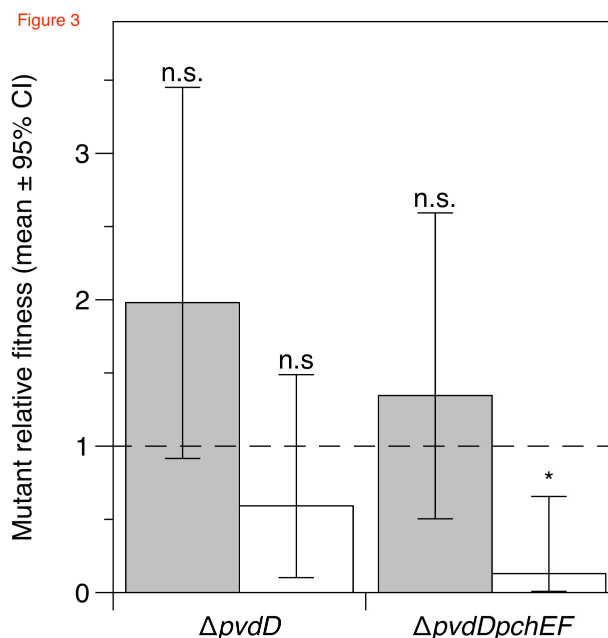
331

332 **Figure 2.** (a,b) Relative fitness of Δ*pvdD* and Δ*pvdD*Δ*pchEF* mutants in pure culture (grey bars) and in mixed
333 culture with an isogenic wild type (white bars) in ASM after (a) 24 and (b) 48 hours of growth. (c) Cheating
334 by Δ*pvdD* over 48 hours of co-culture results in mixed wild type + mutant cultures showing the same reduction
335 in total population density as pure mutant cultures. Bars show means of 9-10 replicates split across two replica
336 experiments, with associated 95% confidence interval. After ANOVA, post-hoc tests were conducted to
337 determine whether each fitted mean value was significantly different from 1: * = $p < 0.02$; n.s. = not significant.

338

339 **Siderophore mutants are outcompeted by the wild type in model of CF bronchiolar biofilm.**
340 ASM models the chemistry of CF mucus (Palmer et al 2007, Turner et al 2015), but liquid culture lacks realistic
341 spatial structure. To allow bacteria to form biofilm associated with bronchiolar surfaces (Bjarnsholt et al 2013),
342 we repeated the monoculture and 50% co-culture experiments in an *ex vivo* model comprising a section of pig
343 bronchiole cultured in ASM for four days. *P. aeruginosa* forms a loose sleeve of mucoid biofilm around the
344 tissue (Harrison and Diggle in press). The only significant predictor of relative fitness was presence/absence
345 of the wild type: both strains had a relative fitness of 1 in pure culture but showed a trend towards being
346 outcompeted in mixed culture (Figure 3). This trend was significant for the Δ*pvdD*Δ*pchEF* mutant.

347



348

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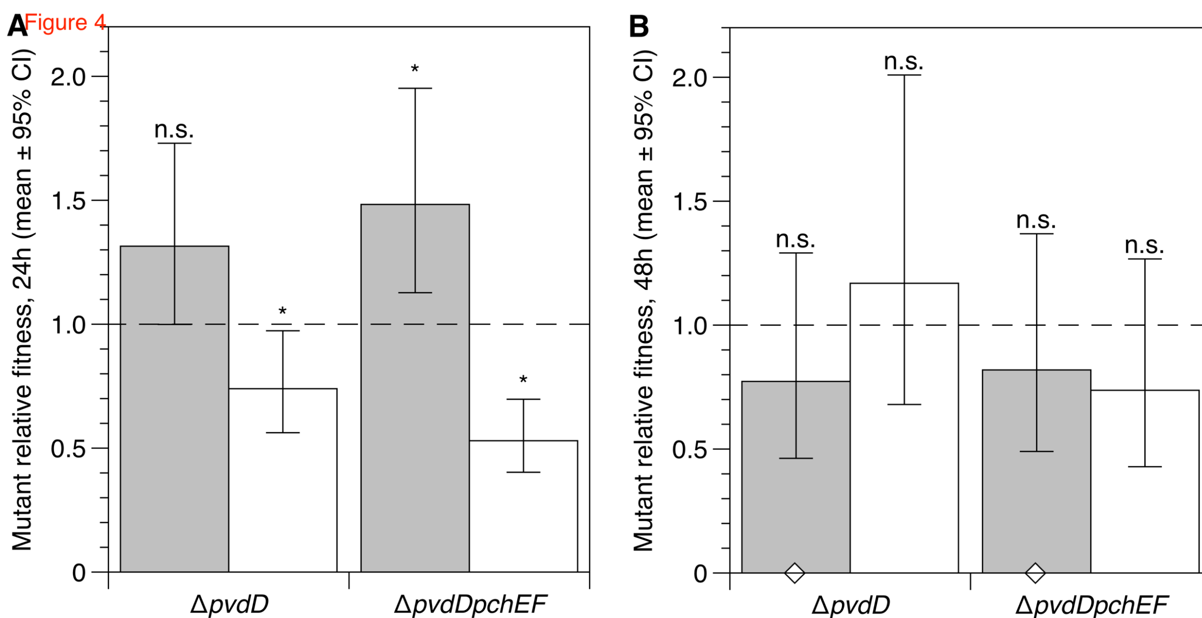
349 **Figure 3.** Relative fitness of Δ*pvdD* and Δ*pvdD*Δ*pchEF* mutants in pure culture (grey bars) and in mixed
350 culture with an isogenic wild type (white bars) in *ex vivo* pig lung + ASM after 96 hours of growth. Bars show
351 means of 8 replicates spread across two replica experiments, with associated 95% confidence interval. After
352 ANOVA, post-hoc *t*-tests were conducted to determine whether each fitted mean value was significantly
353 different from 1: * = $p = 0.007$; n.s. = not significant.
354

355 **Siderophore mutants do not suffer a long-term fitness disadvantage in synthetic wound fluid.**
356 We next repeated the pure/mixed culture experiments in synthetic wound fluid (SWF: Werthén et al 2010).
357 The only significant predictor of relative fitness at 24 hours was presence/absence of the wild type (Figure 4a).
358 The Δ*pvdD* mutant was as fit as the wild type in pure culture, and the Δ*pvdD*Δ*pchEF* mutant slightly and
359 significantly fitter, but both mutants were outcompeted by the wild type in mixed culture. After 48 hours
360 (Figure 4b), there was no difference between genotypes or culture conditions: both mutants had a relative
361 fitness of 1 regardless of wild type presence.



362
363 **Figure 4.** Relative fitness of Δ*pvdD* and Δ*pvdD*Δ*pchEF* mutants in pure culture (grey bars) and in mixed
364 culture with an isogenic wild type (white bars) in SWF after (a) 24 and (b) 48 hours of growth. Bars show
365 means of 10 replicates split across two replica experiments, with associated 95% confidence interval. After
366 ANOVA, post-hoc *t*-tests were conducted to determine whether each fitted mean value was significantly
367 different from 1: * = $p < 0.02$; n.s. = not significant. Confidence intervals and *p*-values in (b) are taken from a
368 model that excluded two outliers where relative fitness was zero (indicated by open diamonds): including these
369 outliers in the analysis did not change any of the conclusions given in the text.
370

371 **Siderophore mutants do not have any fitness disadvantage in synthetic chronic wounds.**
372 As with the CF lung model, we wished to add spatial structure to SWF to better model a soft tissue infection.
373 SWF was solidified with collagen (Werthén et al 2010) and experiments repeated in the resulting solid plugs.

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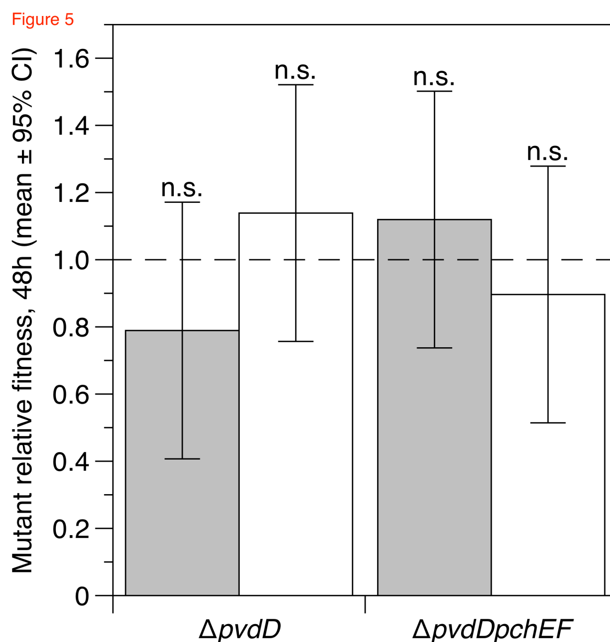
374 After 48 hours in synthetic wounds, (Figure 5), both mutants had a relative fitness of 1 regardless of culture
375 condition.



376

377 **Figure 5.** Relative fitness of Δ*pvdD* and Δ*pvdD*Δ*pchEF* mutants in pure culture (grey bars) and in mixed
378 culture with an isogenic wild type (white bars) in synthetic wounds after 48 hours of growth. Bars show means
379 of 10 replicates split across two replica experiments, with associated 95% confidence interval. After ANOVA,
380 post-hoc *t*-tests were conducted to determine whether each fitted mean value was significantly different from
381 1: n.s. = not significant.
382

383 **Discussion**

384

385 Many experiments have been conducted to explore the social evolution of siderophore production by *P.*
386 *aeruginosa.* These have been used to suggest explanations for the appearance of siderophore mutants in chronic
387 infections; and how this behaviour could be exploited for clinical ends. Our targeted literature review revealed
388 potential restrictions on the generality of predictions made from these experiments about the dynamics of
389 siderophore mutants in nature. First, many experiments used an undefined mutant as a siderophore cheat. We
390 sequenced this strain and found that while it has a deletion of one of the pyoverdine biosynthetic loci, it also
391 carries numerous mutations in genes likely to affect growth and metabolism in unpredictable ways. It is
392 therefore difficult to disentangle the effects of siderophore phenotype vs. other phenotypes on its evolutionary
393 dynamics. Second, most published experiments were carried out in iron-limited minimal broth. Inferences
394 made from these experiments may provide information on the fitness consequences of siderophore loss in
395 acute infections (Granato et al 2016). However, a growing body of evidence strongly suggests that these
396 inferences may not be generalisable to chronic infection contexts, where bacteria experience quite different
397 and idiosyncratic environments that likely affect the cost:benefit ratio of siderophore production, the ability of
398 cells to access each other's siderophores and the growth rate of bacterial populations. Experiments that dissect

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399 the fitness consequences of siderophore loss in environmentally-explicit models of infection could
400 significantly increase our understanding of the natural history of siderophore production, and have enhanced
401 clinical relevance.
402
403 We tested whether deletion mutants for pyoverdine or pyoverdine+pyochelin acted as cheats in four laboratory
404 models designed to represent chronic infections: structured and unstructured models of CF lung infections and
405 non-healing soft-tissue wounds. While these models are not perfect, they have been validated with
406 microbiological and chemical data from real infections, and we present them as examples of improved lab
407 models of specific infection contexts.
408
409 When increased levels of public goods production increase the population growth rate, or increase carrying
410 capacity, cheating mutants are predicted to be under negative frequency-dependent selection (Ross-Gillespie
411 et al 2007). It is therefore usual to conduct cheating assays using a range of starting frequencies, including
412 <50%. We chose to initially conduct cheating assays using only a 50% starting frequency, as both of the
413 mutants we used have been reported to act as cheats under this condition in iron-limited CAA (Kümmerli et al
414 2009b, 2015; Kümmerli and Brown 2010). Further, if mutants that cheat from low starting frequencies cannot
415 maintain this advantage as they become more common, then the likely clinical significance of cheating as a
416 determinant of virulence – and the potential power of a "Trojan horse" approach to managing infection – is
417 called into question. Further investigation of different starting frequencies was not necessary: in the case of
418 ASM, the single mutant showed cheating dynamics even at this high starting frequency, and in all other media,
419 both mutants had equal fitness to the wild type in pure culture so cannot be called cheats regardless of the
420 outcome of competition at any starting frequency.
421
422 Wild-type *P. aeruginosa* produced siderophores in all of our test environments. However, there was
423 environment-dependent variation in siderophore production, and the ratio of pyochelin:pyoverdine was higher
424 in chronic infection models than in CAA (Figure S1). This is consistent with a suggestion by other authors that
425 pyochelin production may be favoured in chronic infection, where iron is more freely available, or is bound to
426 weaker host chelators than transferrin (Dumas et al 2013, Hunter et al 2013, McCallin et al 2015). The
427 pyoverdine mutant was less fit than the wild type in monoculture in artificial CF sputum after 48 hours' growth,
428 demonstrating a benefit to pyoverdine production in this environment, and was able to cheat on the wild type.
429 In all other environments tested, there was no effect of losing pyoverdine production on fitness. Production of
430 pyoverdine in these environments may therefore be a maladaptive response, or may have benefits other than
431 simple growth rate enhancement. There was a non-significant trend towards this mutant being outcompeted
432 by the wild type in *ex vivo* bronchiolar biofilm. This may be due to poor biofilm production, even when iron
433 is plentiful (Banin et al 2005, Harrison and Buckling 2009). The pyoverdine+pyochelin mutant was less fit
434 than the wild type in artificial CF sputum and model bronchiolar biofilms, but had no disadvantage in synthetic
435 wound fluid or synthetic wound biofilm.

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436
437 These results underline the importance of genotype × environment interactions in determining bacterial fitness,
438 and hence the necessity of carefully-designed lab models for predicting the likely consequences of mutations
439 in infection. We previously reported that *P. aeruginosa* QS mutants, which are cheats in an acute infection
440 context (Rumbaugh et al 2009, Wilder et al 2011), are not cheats in an *ex vivo* model of chronic CF lung
441 infection (Harrison et al 2014). Together with the present results, this demonstrates the unreliability of
442 extrapolating predictions about bacterial social evolution from one infection context to another.
443
444 So why do siderophore-null mutants arise during chronic infection? Are they cheats, or simply better adapted
445 to local growth conditions? Are they not selected at all, but present transiently and/or at low frequencies? We
446 cannot answer these questions with current data. Careful choice and optimisation of *in vitro* models that allow
447 for long-term evolution experiments in realistic environments will be invaluable in providing answers.
448 Alongside such models there is also a need for (a) quantitative, rather than qualitative, data from patients on
449 the prevalence of siderophore mutants and (b) more considered choice of “wild type” and mutant genotypes,
450 paying particular attention to genotypes that commonly arise during chronic infection. The fitness
451 conseqeunces of siderophore loss could depend on the nature of the mutations involved, and on background
452 genotype. In future, it would be useful to conduct experiments with a range of clinical isolates that are most
453 typical of those seen in patients, and/or with constructed mutants that recapitulate these.
454
455 For instance, we and other authors have focussed on experiments with siderophore-null mutants that result
456 from mutations in siderophore biosynthesis. However, a recent study of siderophore mutants isolated from CF
457 patients found that the majority actually carried mutations in the regulatory gene *pvdS* (Andersen et al 2015).
458 Sequencing clones from an *in vitro* evolution experiment also revealed pyoverdine-negative phenotypes that
459 stemmed from *pvdS* mutation (Ross-Gillespie et al 2015, R Kümmerli, personal communication). *PvdS*
460 encodes a sigma factor that positively regulates the expression of the pyoverdine biosynthetic loci and is itself
461 up-regulated by iron starvation (Miyazaki et al 1995), but which also positively regulates the expression of
462 other virulence-related exoproducts (Gaines et al 2007, Hunt et al 2002, Ochsner et al 1996, Wilson et al 2001).
463 Longitudinal sampling from patients (Andersen et al 2015) showed that mutations in siderophore receptors
464 only occur once siderophore-null mutants are present. This was interpreted by the authors as evidence for
465 cheating driving the loss of siderophores. But in the absence of explicit tests for cheating by these isolates in
466 co-culture with co-isolated wild types, in growth media that mimics lung biofilm, the longitudinal pattern
467 presented does not unequivocally prove this conclusion. Other possible explanations include early selection
468 on regulatory loci due to other downstream phenotypes and/or reduced transcriptional costs; a relaxation of
469 purifying selection for functional siderophore receptors in the late stages of infection when iron is plentiful; a
470 bias in the data set towards early isolates which increases the conditional probability that later isolates are also
471 siderophore-null; or a combination of all of these factors.
472

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473 Our results demonstrate the necessity of evaluating and modelling in-host environments as carefully as possible
474 if we aim to understand in-host microbiology. Lab experiments in simple *in vitro* conditions have limited
475 ecological and clinical validity and so limited predictive power. A plethora of data on the chemical and
476 microbial ecology of chronic infection is now available, providing abundant material for researchers wishing
477 to study the natural history pathogens in the lab (e.g. for CF: Cowley et al 2015, Flynn et al 2016, Folkesson
478 et al 2012, Kyle et al 2015, Quinn et al 2014, Roberts et al 2015, Turner et al 2015, Yang et al 2011). This
479 information is ripe for consideration by microbiologists who – quite justifiably – see great potential for
480 manipulating the in-host ecology of pathogens in order to halt the progression of debilitating chronic infection.
481
482
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488
489 **Author contributions**
490 FH & SPD conceived the study and designed experiments. FH, AM and ACS carried out experimental work.
491 FH & AM analysed data. FH drafted the manuscript. All authors contributed to manuscript development.
492
493 **Competing financial interests**
494 The authors declare that there are no competing financial interests in relation to the work described.
495
496 **Materials and correspondence**
497 Correspondence and requests for materials should be addressed to FH and SPD.
498
499
500

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732 # Supplementary Information

733

734 **SuppInfo_1.xls**. Information on the SNPs and deletion found in the genome of PAO6609 (PAO9) (Figure 1).

735 Will be supplied on acceptance or during review process

736

737 **SuppInfo_2.xls**. Raw data for Figures 2-5, S1-2. Will be supplied on acceptance or during review process

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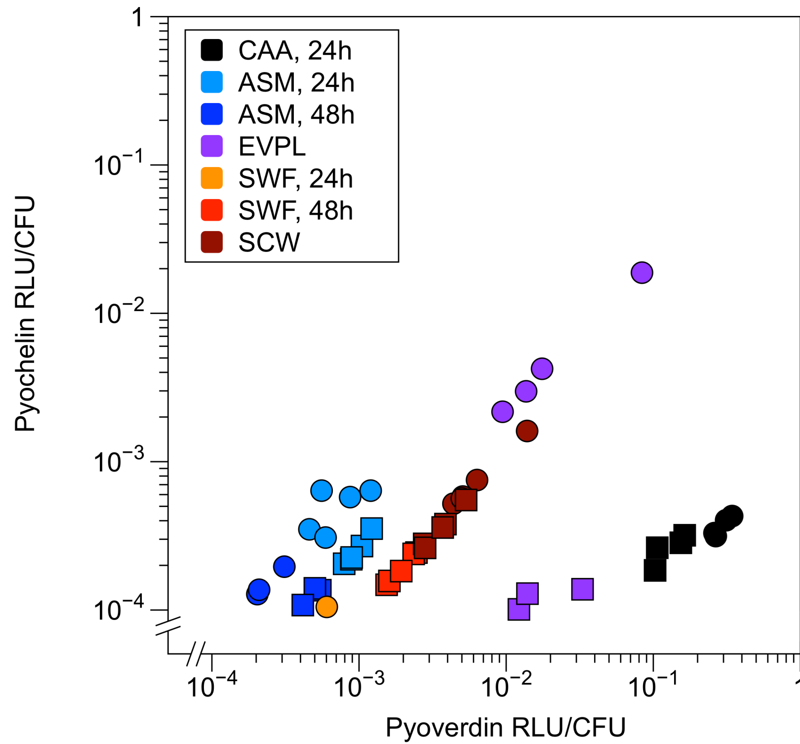

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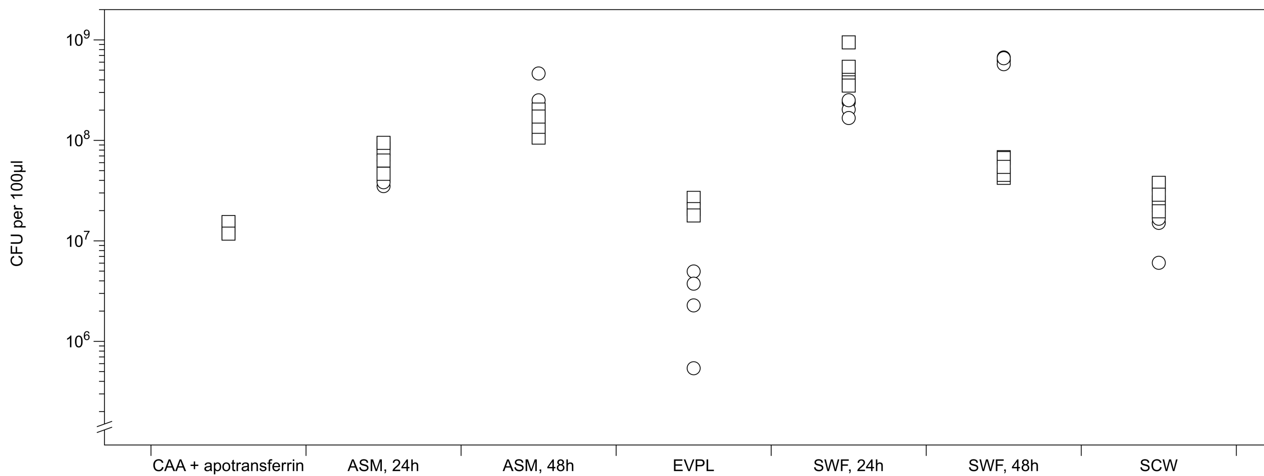

740

741 **Figure S1.** (a) Levels of pyoverdin and pyochelin produced by wild-type *P. aeruginosa* in the different 742 environments explored in this study. Molecules were detected by excitation-emission assays of 100 µl aliquots 743 of culture supernatant and expressed as relative luminescence units (RLU) divided by the number of *P.* 744 *aeruginosa* colony-forming units (CFU) present in 100 µl of the original culture. Symbols denote experimental 745 block. (b) Bacterial density (CFU per 100 µl) the different environments. Symbols denote experimental block.

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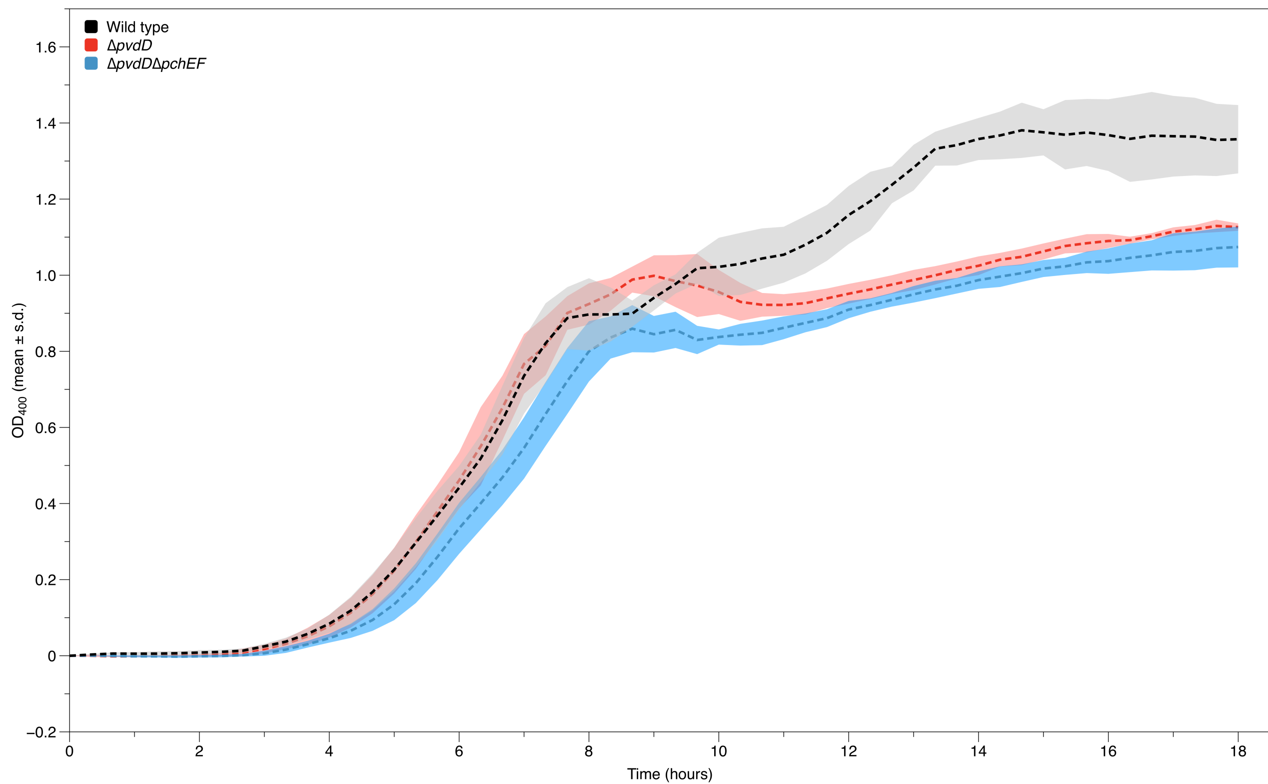
746 CAA: casamino acids medium, ASM: artificial sputum medium, EVPL: *ex vivo* pig lung model, SWF:
747 synthetic wound fluid, SCW: synthetic chronic wound model.
748



749
750 **Figure S2.** Growth of wild type (black), ΔpvdD (red) and ΔpvdDΔpchEF (blue) P. aeruginosa in 200 µl ASM
751 over 18 hours. Optical density was read at 400 nm to minimise interference from pyoverdine absorbance in
752 wild-type cultures. Lines show means of four replica cultures, shaded areas show ± one standard deviation.

Table 1. Results of a review of the empirical literature on *P. aeruginosa* siderophore mutants and cheating.

Reference	Producer strain	Non-producer strain	Growth medium	Specific test for cooperation	Location of results in publication	Non-producer start frequency	Cheating observed?	Notes
Griffin et al 2004	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Text	0.5	Variable	Cheating was concluded on the basis of differences in density of pure vs. mixed culture, relative fitness not reported.
Harrison et al 2006	ATCC 15692 (PAO1)	PA06609 (PAO9)	Waxworm	Yes	Figs 3, 4	0.03-0.9	No	Highest reported cheat relative fitness is 1, at start frequencies ≤ 0.01
Ross-Gillespie et al 2007	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Fig 3	0.001-0.99	Variable	Cheating observed at starting frequencies ≤ 0.1; total population density only affected at lowest start frequency
Ross-Gillespie et al 2007	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	Yes	Fig 3	0.001-0.99	Variable	Cheating observed at starting frequencies of 0.001, but no effect on total population density. This mutant gains less fitness benefit from coculture than PAO9 does
Ross-Gillespie et al 2007	UCBPP-PA14 (clinical)	Spontaneous mutant	CAA + apotransferrin	Yes	Fig 3	0.001-0.99	Variable	Cheating observed at start frequencies ≤ 0.1, but no effects on total population density. This mutant gains less fitness benefit from coculture than PAO9 does
Harrison et al 2008	PAO985	Evolved clones	CAA + apotransferrin / iron, ± *S. aureus*	No	Figs 1,2	0		
Kümmerli et al 2009a	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Text	0.33	Yes	
Kümmerli et al 2009b	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	No				
Kümmerli et al 2009b	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*/Δ*pchEF*	CAA + apotransferrin	Yes	Fig 4	0.17-0.83	Yes	Cheat is always fitter than the wild type, even when competed at a starting frequency of 0.83.
Kümmerli et al 2009c	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Fig 2	0.33	Variable	Cheating is time dependent
Harrison and Buckling 2009	ATCC 15692 (PAO1)	*pvdF* transposon mutant in MPAO1 background (PA2396-C04::IS-lacZ/hah)	CAA + apotransferrin	No, but raw data available for re-analysis	Raw data	0.5	No	Re-analysis of raw data reveals this mutant is less fit then the wild type in mixtures with a starting frequency of 0.5 in planktonic and biofilm culture.
Harrison and Buckling 2009	ATCC 15692 (PAO1)	Clones evolved from PAO6049	CAA + apotransferrin	No	Raw data	0.5	No	Mutants outcompete the wild type in planktonic mixed culture, but so does their siderophore-pruducing ancestor & they are lab adapted, growing as well as PAO1 in pure culture.
Ross-Gillespie et al 2009	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Fig 2	0.09	No	

Ross-Gillespie et al 2009	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	Yes	Fig 2	0.09	Variable	Cheating observed at high cell density only.
Ross-Gillespie et al 2009	UCBPP-PA14 (clinical isolate)	Spontaneous mutant	CAA + apotransferrin	Yes	Fig 2	0.09	Variable	Cheating observed at high cell density only.
Kümmerli et al 2010	ATCC 15692 (PAO1)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Text	0.5	Yes	
Kümmerli and Brown 2010	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	Yes	Fig 5	0.5	Yes	
Kümmerli and Brown 2010	PAO6049	PA06609 (PAO9)	CAA + apotransferrin	No				
Kümmerli and Brown 2010	Environmental isolate	Spontaneous mutant	CAA + apotransferrin	Yes	Fig 5	0.5	Yes	
Jiricny et al 2010	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	Yes	Figs 4,5	0.09	Yes	
Jiricny et al 2010	ATCC 15692 (PAO1)	PAO1 Δ*pchEF*	CAA + apotransferrin	No		0.09		
Jiricny et al 2010	ATCC 15692 (PAO1)	PAO1 Δ*pvdD*/Δ*pchEF*	CAA + apotransferrin	No		0.09		
Jiricny et al 2010	11 various isolates	Spontaneous mutants	CAA + apotransferrin	Yes	Figs 4,5	0.09	Variable	Cheating observed for 8/11mutants. Three non-cheats (with high pyoverdin production) may be driving the reported trend.
Harrison and Buckling 2011	PAO6049	Evolved clones	CAA + apotransferrin	Yes	Fig 1	0.05, 0.5	Variable	Cheating observed at low starting frequencies for a minority of mutants (mean relative fitness = 1)
Harrison and Buckling 2011	PAO1 Δ*mutS*	Evolved clones	CAA + apotransferrin	Yes	Fig 1	0.05, 0.5	Variable	Cheating observed at low starting frequencies only for a majority of mutants, but some can cheat from a starting frequency of 0.5.
Dumas and Kümmerli 2012	ATCC 15692 (PAO1)	Evolved clones	CAA + apotransferrin	No	Figs 3, 4	0		Pyoverdine-deficient clones evolved, but their presence did not consistently reduce population growth - some were associated with increased growth.
Dumas et al 2013	ATCC 15692 (PAO1)	PAO1 Δ*pvdD* and Δ*pvdD*/Δ*pchEF*	CAA + apotransferrin	No	Fig 4	Three-strain mix, each strain at 0.33		Carbon source, pH and temperature determine the relative growth advantage conferred by siderophores in monoculture. In acidic pH, Δ*pvdD* grows better. Report outcome of simuated competitions based on monoculture growth parameters.
Ghoul et al 2014	Cystic fibrosis isolate	2x spontaneous mutants	CAA	Yes	Figs 3,4	0.1	Yes	Mutant with reduced pyoverdine production cheats on parent strain, and is cheated on by a second spontaneous mutant with even lower pyoverdine production.
Ross-Gillespie et al 2015	PAO1 Δ*pvdD*	PAO1 Δ*pvdD*/Δ*pchEF*	CAA + apotransferrin	Yes	Fig 2	0.2	Yes	This experiment competed a double pyoverdine/pyochelin knockouts against a single pyoverdine knockout.

Ross-Gillespie et al 2015	PAO1 Δ*pchEF*	PAO1 Δ*pvdD*/Δ*pchEF*	CAA + apotransferrin	Yes	Fig 2	0.2	Yes	This experiment competed a double pyoverdine/pyochelin knockouts against a single pyochelin knockout.
Kümmerli et al 2015	ATC 15692 (PAO1)	PAO1 Δ*pvdD*	CAA + apotransferrin	No, but can be inferred from data supplied	Figs 1, 2, 3	0.5	Yes	There is a coevolutionary arms race, whereby producers become less exploitable and non-producers become better cheats. Non-producer frequency is negatively correlated with population growth. Non-producers grow less well than producers in monoculture, and contemporary pairs show relative fitness of non-producers is >1 (though graphs suggest this effect is small).
Andersen et al 2015	Cystic fibrosis isolates	CF isolates	No culture	No	Text			Cheating inferred by sequence of mutations affecting pyoverdine production and uptake. Very few mutations reported in pyoverdine biosynthetic loci and none pyochelin loci. Most mutations are in pvdS.
Ghoul et al 2016	ATCC 15692 (PA01)	PA06609 (PAO9)	CAA + apotransferrin	Yes	Figs 1a, 2a	0.02-0.10	Variable	Mutant cheats only if added to producer cultures before the onset of stationary phase.