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1 *Pseudomonas aeruginosa* isolates co-incubated with *Acanthamoeba castellanii*
2 exhibit phenotypes similar to chronic cystic fibrosis isolates

3 Short title: Effects of adaptation to amoeba on phenotypes expressed by *P.*
4 *aeruginosa*

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21 ## Abstract

22 The opportunistic pathogen, *Pseudomonas aeruginosa,* is ubiquitous in the environment, and in
23 humans is capable of causing acute and chronic infections. *P. aeruginosa,* when co-incubated
24 with the bacterivorous amoeba, *Acanthamoeba castellanii,* for extended periods, produced
25 genetic and phenotypic variants. Sequencing of late-stage amoeba-adapted *P. aeruginosa*
26 isolates demonstrated single nucleotide polymorphisms within genes that encode known
27 virulence factors, and this correlated with a reduction in expression of virulence traits. Virulence
28 towards the nematode, *Caenorhabditis elegans,* was attenuated in late-stage amoeba-adapted *P.*
29 *aeruginosa* compared to early stage amoeba-adapted and non-adapted counterparts. Late-stage
30 amoeba-adapted *P. aeruginosa* lost competitive fitness compared to non-adapted counterparts
31 when grown in nutrient rich media. However, non-adapted *P. aeruginosa* were rapidly cleared
32 by amoeba predation, whereas late-stage amoeba-adapted isolates remained in higher numbers
33 24 h after ingestion by amoeba. In addition, there was reduced uptake by macrophage of
34 amoeba-adapted isolates and reduced uptake by human neutrophils as well as increased survival
35 in the presence of neutrophils. Our findings indicate that the selection imposed by amoeba on *P.*
36 *aeruginosa* resulted in reduced virulence over time. Importantly, the genetic and phenotypic
37 traits possessed by late-stage amoeba-adapted *P. aeruginosa* are similar to what is observed for
38 isolates obtained from chronic cystic fibrosis infections. This notable overlap in adaptation to
39 different host types suggests similar selection pressures among host cell types.

40 ## Author Summary

41 *Pseudomonas aeruginosa* is an opportunistic pathogen that causes both acute infections in plants
42 and animals, including humans and also causes chronic infections in immune compromised and

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43 cystic fibrosis patients. This bacterium is commonly found in soils and water where bacteria are
44 constantly under threat of being consumed by the bacterial predators, protozoa. To escape being
45 killed, bacteria have evolved a suite of mechanisms that protect them from being consumed or
46 digested. Here we examined the effect of long-term predation on the genotype and phenotypes
47 expressed by *P. aeruginosa.* We show that long-term co-incubation with protozoa resulted in
48 mutations in the bacteria that made them less pathogenic. This is particularly interesting as we
49 see similar mutations arise in bacteria associated with chronic infections. Thus, predation by
50 protozoa and long term colonization of the human host may represent similar environments that
51 select for similar losses in gene functions.

52

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53 **Introduction**

54 Many virulence traits of microorganisms are regulated in response to the environment in order to
55 invade a host, obtain resources, defend against predation by heterotrophic protists, or establish a
56 replication niche. The evolution of virulence, i.e. harm caused by a pathogen towards its host, is
57 a long-standing subject of investigation with important implications for human health. Most
58 opportunistic pathogens are not transmitted person to person but rather transit through the
59 environment between hosts and therefore, it is unlikely that virulence traits evolve in the host (1-
60 3). Rather, it is more likely that these traits evolve in the environment.

61 Predation by protists, or protozoa, is a major mortality factor for bacteria in the environment (4).
62 Virulence traits, particularly those that cause human disease, are hypothesized to have evolved in
63 response to and are maintained by predation pressure, which supports the "coincidental
64 evolution" hypothesis. This hypothesis states that virulence is a coincidental consequence of
65 adaptation to other ecological niches (5-7). Coincidental evolution is supported by examples of
66 factors that play roles in both grazing resistance and virulence towards mammalian hosts (7-9),
67 including traits such as cell-surface alterations, increased swimming speed, toxin release and
68 biofilm formation (5, 7). Conversely, virulence traits may be attenuated or lost when organisms
69 adapt to form a more commensal relationship with a host (10-12). Microorganisms may also
70 develop specific virulence traits against a specific host becoming a specialist pathogen.
71 Although there are many hypotheses for how virulence traits evolve, there have been few studies
72 on the adaptation of specific virulence traits to different host types and environments. Such
73 studies are particularly important for understanding how opportunistic pathogens evolve (13).

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74 *Pseudomonas aeruginosa* is a versatile opportunistic pathogen found in a wide variety of natural
75 habitats. *P. aeruginosa* has a large (6.3 Mb) genome containing many genes for metabolism and
76 antibiotic resistance (14), and coupled with a complex regulatory network allows it to effectively
77 survive in a variety of niches. *P. aeruginosa* is an important pathogen, responsible for both acute
78 nosocomial infections (15) and chronic infections in leg ulcers and particularly in the lungs of
79 cystic fibrosis (CF) patients (16). In the CF lung, it has been shown to evolve towards a more
80 commensal lifestyle by altering the expression of acute virulence traits such as motility, quorum
81 sensing and toxin production (17). While there are many studies addressing the evolution of *P.*
82 *aeruginosa* in the CF lung (17-19), there is less known about the impact of protozoan predation
83 on the evolution of virulence. To address this lack of knowledge, this study investigated the
84 adaptation of *P. aeruginosa* during long-term co-incubation with the amoeba predator,
85 *Acanthamoeba castellanii*. *P. aeruginosa* was co-incubated with *A. castellanii* for 42 days and
86 the impact of co-incubation assessed using a range of phenotypes, including virulence in a
87 *Caenorhabditis elegans* infection model. Adapted populations were also sequenced to
88 investigate the range of mutations that occurred during co-incubation.

89 Results

90 Genotypic changes in adapted strains

91 The number of synonymous and non-synonymous single nucleotide polymorphisms (sSNPs and
92 nsSNPs, respectively) occurring in amoeba-adapted and non-adapted populations were
93 determined. There were 54 nsSNPs and 17 sSNPs detected in the 42 d adapted populations and

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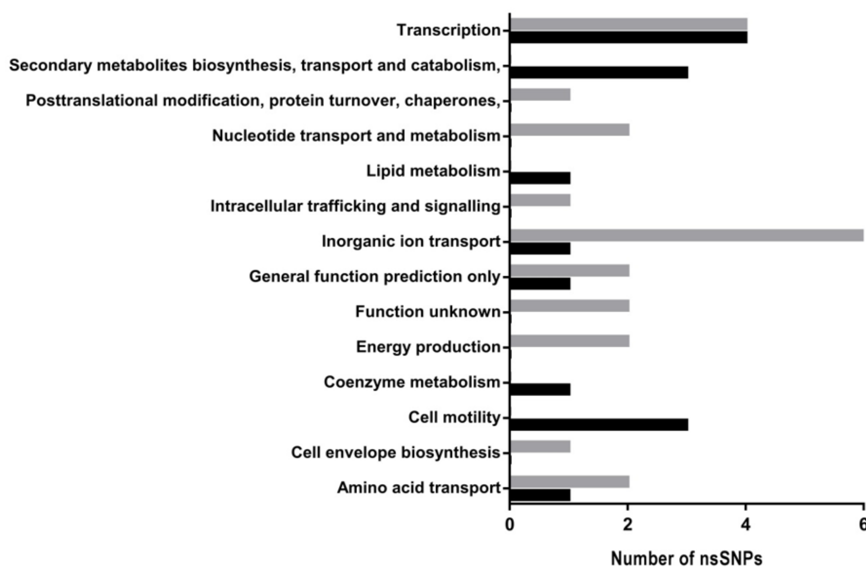
94 65 nsSNPs and 19 sSNPs detected in the 42 d non-adapted populations compared to the parent
95 strain. The genes that contained nsSNPs were grouped into gene functions based on Clusters of
96 Orthologous Groups (COGs) and were distributed into 14 COG categories (Fig 1). Mutations in
97 genes involved in transcription occurred in equal numbers in adapted and non-adapted
98 populations. In contrast, nsSNPs in coenzyme metabolism, lipid metabolism, cell motility and
99 secondary metabolite production occurred solely within the amoeba-adapted population, while
100 COGs representing inorganic ion transport and energy production were over-represented in the
101 non-adapted population (Fig 1).



102

103 **Fig 1. Gene classifications.** Classification of genes containing nsSNPs grouped by COG
104 functional class for amoeba-adapted (black bars) and non-adapted (grey bars) populations.

105 The following nine genes were independently mutated in all three amoeba-adapted replicate
106 populations: the virulence regulator *vreA*, chemotaxis genes *pctB* and PA3349,

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107 lipopolysaccharide biosynthetic gene *lpxO2*, *ppiA* and *polA* involved in translation, a cytochrome
108 oxidase *ccpR,* the siderophore *fvbA*, and the hypothetical protein PA3638. The mutations with
109 the highest frequency in the amoeba-adapted populations occurred within motility genes,
110 however mutations in different genes were responsible for the loss of motility observed in the
111 replicate experimental populations. For example, in population 1, 55.17 % of the reads in the
112 *flgF* gene and 34.63 % of the reads in *flgH* contained a nsSNP. Amoeba-adapted populations 2
113 and 3 contained considerable variation in the gene encoding *flgK,* where 42.31 % of the *flgK*
114 reads from population 2 contained nsSNPs and 96.43 % of *flgK* reads from population 3
115 contained a gene deletion. In addition to flagellar-mediated motility, all 42 d amoeba-adapted
116 populations contained deletions or SNPs within genes associated with twitching motility,
117 specifically 39.53 % of reads encoding *pilN*, 46.43 % reads encoding *pilM* and 25.53 % of reads
118 encoding *pilT* were detected in population 1, 2 and 3 respectively. Further details about the gene
119 function, frequency of SNPs and codon/amino acid substitution can be found in Table 1.

120 Analysis using DAVID functional tools predicted that the second unique biological process
121 affected by SNPs as a result of long-term adaption with amoeba is the biosynthesis, transport and
122 catabolism of secondary metabolites. All 42 d amoeba-adapted populations contain SNPs in
123 *pvdJ* or *pvdD* and these mutations are predicted to affect the synthesis of pyoverdine.
124 Additionally, in an analysis of the 42 d amoeba-adapted populations, *yfiH* and *yafE* were also
125 categorized under secondary metabolite synthesis (20). Non-synonymous SNPs within these
126 genes may further prevent synthesis of secondary metabolites.

127 **Effect of co-incubation of *P. aeruginosa* with *A. castellanii* on motility**

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128 The mutations with the highest frequency in the amoeba-adapted populations occurred within
129 motility genes. Thus, swimming, swarming and twitching motility of the isolates from adapted
130 and non-adapted populations were compared. The long-term co-incubation of *P. aeruginosa*
131 with amoeba resulted in a reduction in twitching motility (Fig 2a; $F_{2, 534} = 295.1$, $p < 0.001$). *P.*
132 *aeruginosa* isolates from the 3 d amoeba-adapted and non-adapted populations did not differ
133 significantly ($p = 0.53$), however, after 24 d twitching was significantly reduced compared to the
134 non-adapted isolates ($p < 0.001$) and in the 42 d population, the mean twitching motility of
135 amoeba-adapted isolates was 10-fold less than isolates that were incubated in the absence of *A.*
136 *castellanii* ($p < 0.001$).

137 Co-incubation with *A. castellanii* also resulted in a decrease in swimming motility (Fig 2b; $F_{2, 534}$
138 $= 15.6$, $p < 0.001$), where *P. aeruginosa* isolates from 3 d amoeba-adapted populations had a
139 swim area of half that of isolates from non-adapted populations ($p < 0.001$). This pattern of
140 reduced swimming motility was observed for amoeba-adapted isolates from the 24 and 42 d
141 populations as well ($p < 0.001$ and $p < 0.001$, respectively).

142 *P. aeruginosa* isolates demonstrated a reduction in swarming motility as a result of co-incubation
143 with amoeba, which varied over time in a non-linear fashion (Fig 2c; $F_{2, 534} = 7.597$, $p < 0.001$).
144 Swarming was significantly reduced in isolates from amoeba-adapted populations at days 3 and
145 24 ($F_{1, 534} = 21.73$, $p < 0.001$). Post hoc analysis shows that after 3 d of co-incubation the
146 swarming distance of non-adapted isolates of *P. aeruginosa* was twice that of amoeba-adapted
147 isolates ($p > 0.001$). After 24 d of co-incubation the swarming distance exhibited by *P.*
148 *aeruginosa* isolates derived from amoeba-adapted and non-adapted populations were further

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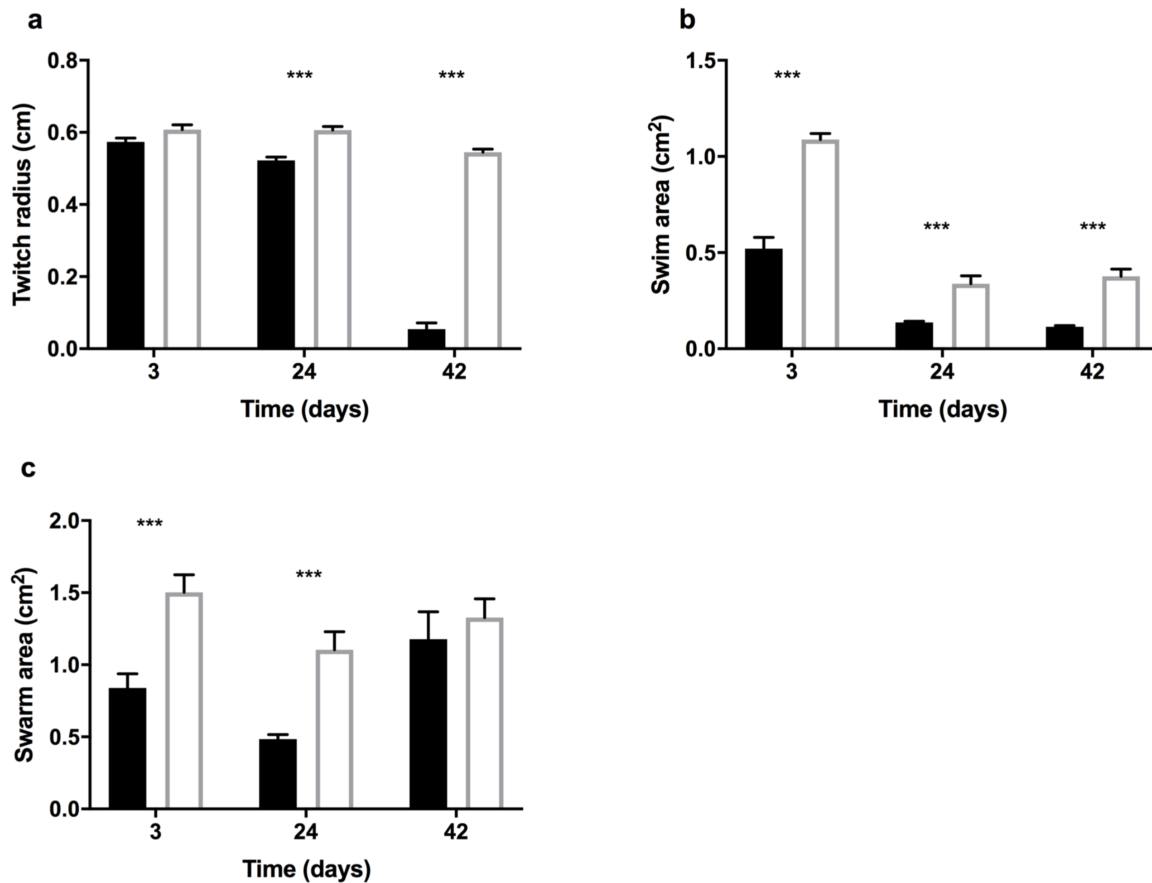
149 reduced, however, there is still a significant reduction in swarming of amoeba-adapted isolates
150 compared to non-adapted isolates ($p < 0.001$). After 42 d there was no significant difference
151 between the average swarming motility of isolates from either population ($p = 0.189$).



152

153 **Fig 2. Effect of co-incubation of *P. aeruginosa* with *A. castellanii* on motility.** Twitching (a),
154 swimming (b) and swarming motility (c) of *P. aeruginosa* isolates derived from amoeba-adapted
155 (closed) and non-adapted (open) isolates on days 3, 24 and 42. Data are presented as Means ±
156 SEM. *** $p < 0.001$.

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157 **Biofilm formation and planktonic growth of isolates from adapted populations**

158 As flagella and pili also impact biofilm formation, biofilm biomass and planktonic growth rates
159 of adapted and non-adapted isolates were compared. Co-incubation of *P. aeruginosa* with *A.*
160 *castellanii* had a significant effect on *P. aeruginosa* surface colonization (Fig 3a; $F_{2, 354} = 15.7$, p
161 < 0.001). Post hoc analysis revealed no difference between treatments after 3 d ($p = 0.998$).
162 However, *P. aeruginosa* from amoeba-adapted day 24 populations formed 10-fold less biofilm
163 than isolates from non-adapted day 24 populations ($p < 0.001$). Although the average biomass
164 of biofilms formed by the amoeba-adapted isolates increased after 42 d of co-incubation with
165 amoeba, biofilm biomass remained 2-fold lower than that of the non-adapted population ($p <$
166 0.001). Additionally, the presence of amoeba exerted a strong negative effect on the planktonic
167 growth of *P. aeruginosa* isolates derived from the amoeba-adapted population (Fig 3b; $F_{1, 354} =$
168 29.6, $p < 0.001$). The planktonic growth rate of *P. aeruginosa* after 3 d was the same regardless
169 of the population ($p = 0.56$). However, after 24 and 42 d of amoebal-driven selection the
170 planktonic growth rate of amoeba-adapted derived isolates was significantly less than the non-
171 adapted isolates ($p < 0.05$ and $p < 0.001$ respectively).

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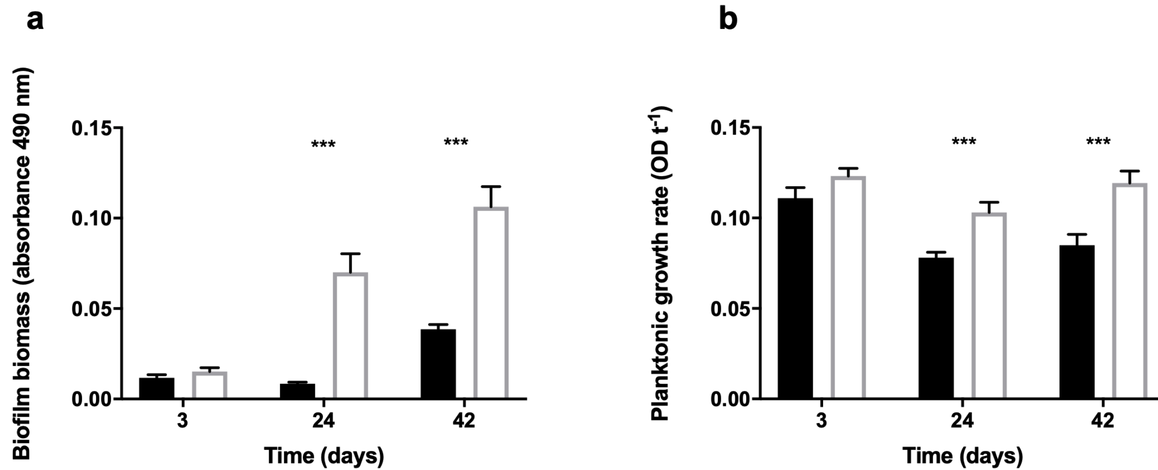

172

173 **Fig 3. Effect of co-incubation of *P. aeruginosa* with *A. castellanii* on biofilm formation and**
174 **growth.** Biofilm biomass (a) and planktonic growth rates (b) of *P. aeruginosa* amoeba-adapted
175 (closed) and non-adapted (open) isolates obtained from 3, 24 and 42 d populations when grown
176 in LB_{10} media at 37°C. Data are presented as Means ± SEM. *** $p < 0.001$.

177 **Quantification of pyoverdine and rhamnolipids**

178 *P. aeruginosa* isolates obtained after co-incubation with *A. castellanii* produced reduced
179 quantities of pyoverdine compared to isolates from non-adapted populations (Fig 4a; $F_{1, 174} =$
180 45.74, $p < 0.001$). Although pyoverdine production was reduced in both amoeba-adapted and
181 non-adapted populations ($F_{2, 174} = 12.08$, $p < 0.001$), the concentration of pyoverdine in
182 supernatants from amoeba-adapted isolates from 3 d populations was reduced 2-fold compared to
183 non-adapted isolates ($p < 0.001$) and was further reduced after 24, and 42 days of selection ($p <$
184 0.001).

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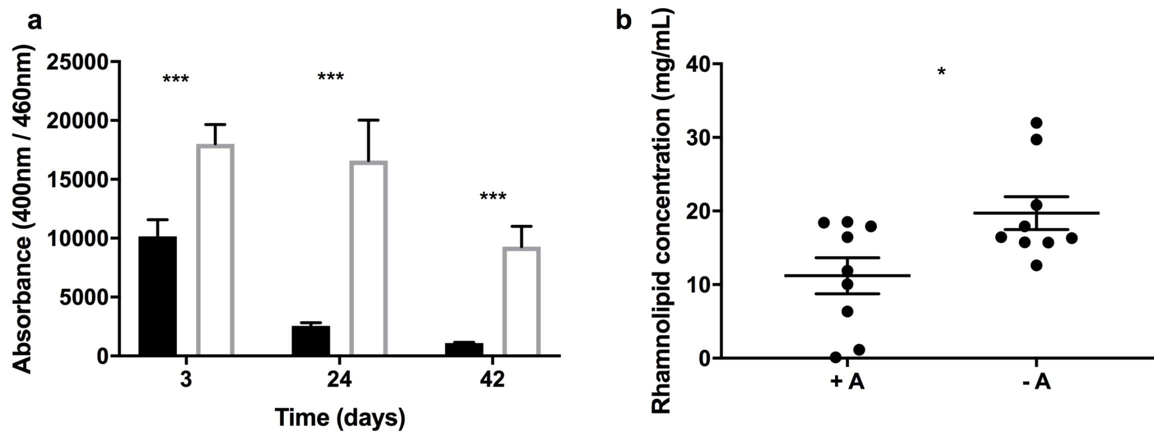
185 Rhamnolipid production varied within the 42 d amoeba-adapted and non-adapted isolates but
186 amoeba-adapted isolates produced less rhamnolipid overall when compared with the non-adapted
187 population (Fig 4b; $t_{16} = 2.571$, $p = 0.0205$).



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189 **Fig 4. Effect of co-incubation of *P. aeruginosa* with *A. castellanii* on pyoverdine and**
190 **rhamnolipid production.** Quantification of pyoverdine (a) in supernatants from *P. aeruginosa*
191 isolates obtained from amoeba-adapted (closed) and non-adapted (open) populations from days
192 3, 24 and 42. Quantification of rhamnolipids (b) in supernatants from *P. aeruginosa* isolates
193 obtained from day 42 amoeba-adapted (+A) and non-adapted (– A) isolates using the orcinol
194 method, with a correction factor of 2.5. Data are presented as Means ± SEM. * $p < 0.05$ *** $p <$
195 0.001.

196 **Amoeba-adapted *P. aeruginosa* are more competitive than non-adapted isolates when**
197 **grown with amoeba**

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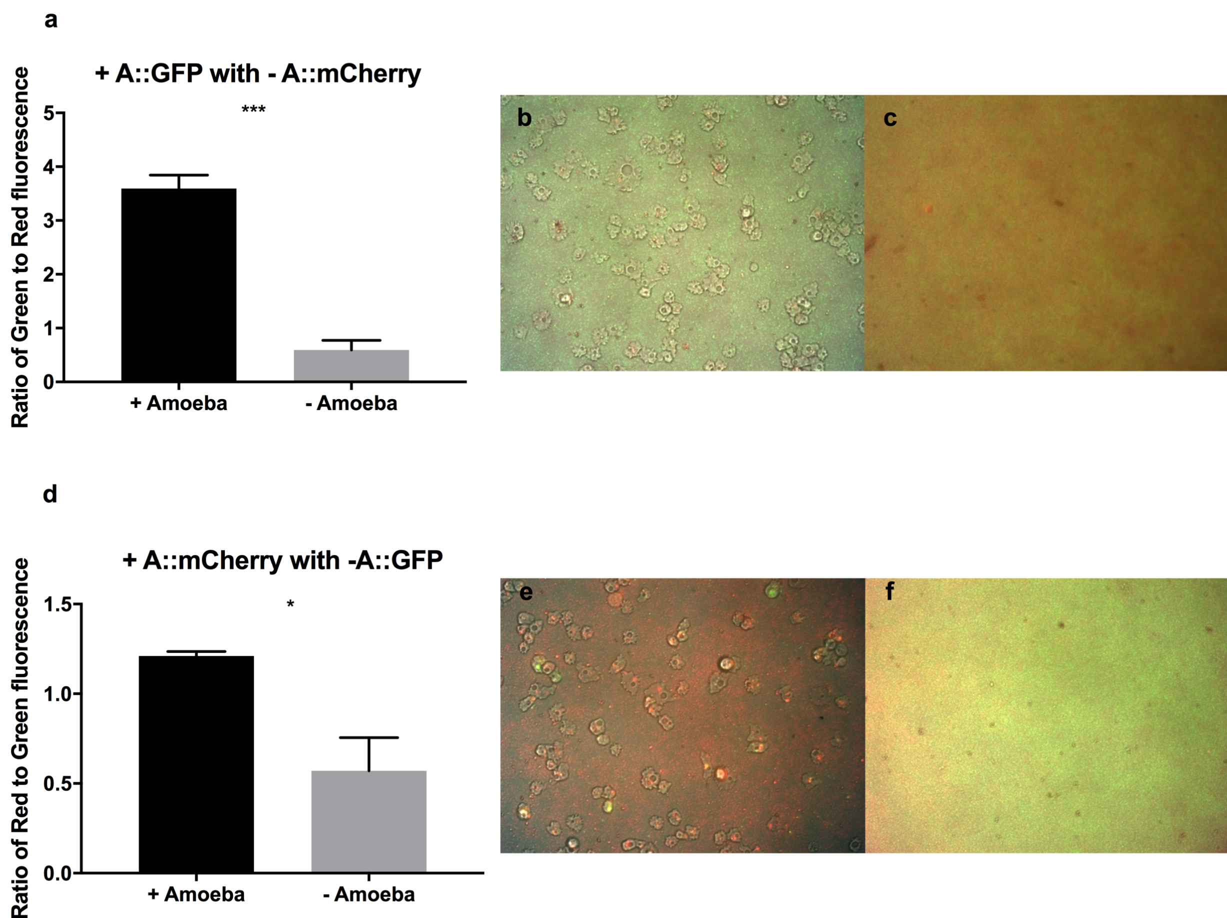
198 To investigate whether adaptation with amoeba confers a fitness advantage to *P. aeruginosa*
199 when grown with amoeba, we mixed fluorescent-tagged amoeba-adapted and non-adapted
200 isolates and grew them together with amoeba. After 48 h co-incubation with amoeba, the
201 proportion of amoeba-adapted cells is always higher when both amoeba-adapted::GFP (Fig 5a;
202 $F_{1,4} = 95.27$, $p = 0.000617$) and amoeba-adapted::mCherry (Fig 5b; $F_{1,4} = 11.85$, $p = 0.0262$) are
203 competed with the reciprocally tagged non-adapted strain, compared to no amoeba controls.



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205 **Fig 5. Competition of amoeba-adapted and non-adapted *P. aeruginosa* grown with amoeba**

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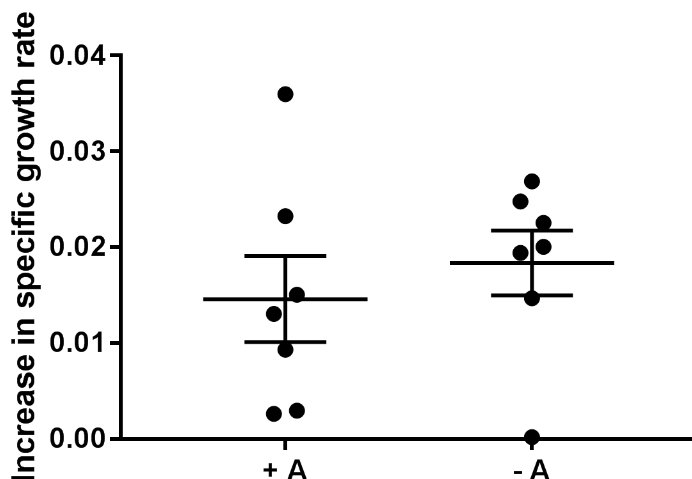
206 The fluorescence ratios of Day 42 + A::GFP *P. aeruginosa* mixed with – A::mCherry (a,b,c) and
207 + A::mCherry with – A::GFP (d,e,f) after 48h of incubation with (black bars, b, e) and without
208 (grey bars, c, f) *A. castellanii*. Data are presented as Means ± SEM. * $p < 0.05$, *** $p < 0.001$.

209 **Growth of amoeba-adapted and non-adapted *P. aeruginosa* in media or media**
210 **supplemented with amoeba supernatant**

211 To investigate whether the amoeba-adapted strains were utilizing amoeba secretions to out-
212 compete the non-adapted strains, 9 randomly selected amoeba-adapted and non-adapted isolates
213 were grown with and without the addition of amoeba supernatant to the growth media. The
214 addition of amoeba supernatant to the growth media resulted in specific growth rate increases of
215 -0.02 to 0.04 by 42 d amoeba-adapted and non-adapted *P. aeruginosa* isolates compared to the
216 minimal media supplemented with the same amount of glucose. There was no significant
217 difference in growth between amoeba-adapted and non-adapted isolates when grown in amoeba
218 supernatant (Fig 6; $t_{12} = 0.5147$, $p = 0.5147$).



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220 **Fig 6. Growth of amoeba-adapted and non-adapted isolates in media supplemented with**
221 **amoeba supernatant.** Increase in specific growth rate of day 42 amoeba-adapted and non-
222 adapted *P. aeruginosa* populations in M9 salts + 0.04% glucose supplemented with amoeba
223 supernatant compared to no addition (n=9). Data are presented as Means ± SEM. No significant
224 difference was observed in amoeba-adapted isolates.

225 **Amoeba-adapted *P. aeruginosa* isolates exhibit reduced uptake by and enhanced survival**
226 **within amoeba and macrophages**

227 Since the enhanced fitness of amoeba-adapted isolates in the presence of amoeba was not due to
228 increased growth rate, the intracellular survival of 42 d amoeba-adapted and non-adapted
229 populations were determined using a modified gentamicin protection assay. Intracellular CFUs 3
230 h after infection of non-adapted isolates within amoeba were higher than amoeba-adapted CFUs,
231 however, after 24 h the numbers of surviving intracellular non-adapted cells had decreased and
232 were comparable to the amoeba-adapted numbers (Fig 7a; Adaptation×Time $F_{1,32} = 14$, $p <$
233 0.001). The same trend was observed when the assay was conducted with raw 264.7
234 macrophages. There was a significant interaction of amoeba adaptation and incubation time (Fig
235 7b; Adaptation×Time $F_{4, 64} = 6.692$, $p < 0.001$), with a higher initial uptake of 42 d non-adapted
236 populations compared to the amoeba adapted strains, resulting in higher initial intracellular CFU
237 counts, followed by a constant decrease in viable intracellular numbers between 5 and 18 h post-
238 infection. The 42 d amoeba-adapted populations were taken up by macrophage in lower initial
239 numbers, and the number of viable intracellular CFUs did not decrease to the same extent as the
240 non-adapted populations, resulting in comparable numbers at 18 h post-infection. At 24 h post-

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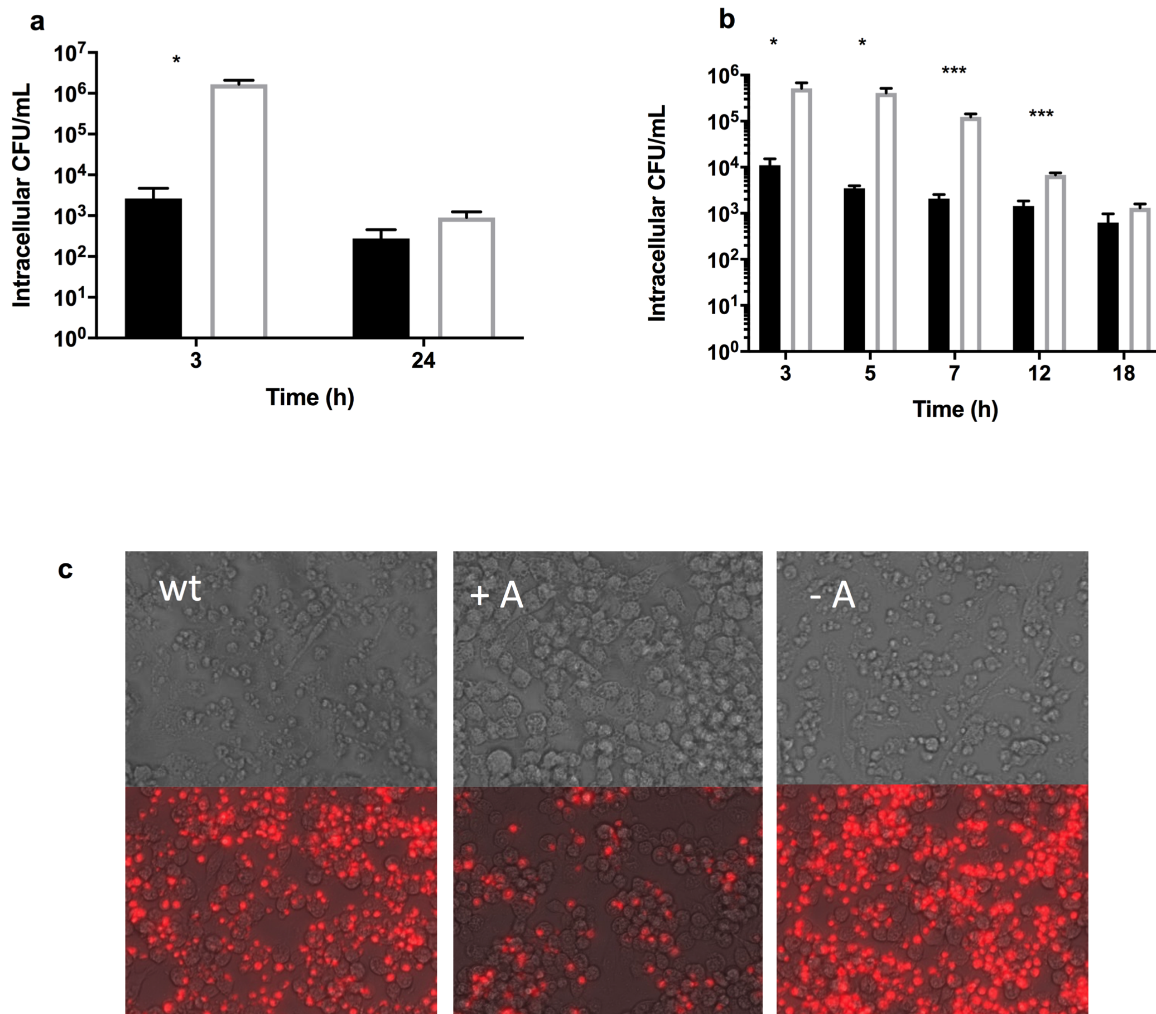
241 infection, macrophage cells infected with non-adapted *P. aeruginosa* exhibited morphological
242 changes and appeared similar to those infected with the wild type strain (Fig 7c). Propidium
243 iodide staining showed that many of these macrophages were dead. In contrast, macrophage
244 infected with amoeba-adapted *P. aeruginosa* exhibited a more normal morphology, with fewer
245 cells taking up the propidium iodide stain.



246

247 **Fig 7.** Intracellular survival of day 42 amoeba-adapted (closed) and non-adapted (open) strains
248 in CFU ml^{-1} over time in a modified gentamicin protection assay (log-scale, n=3) conducted with

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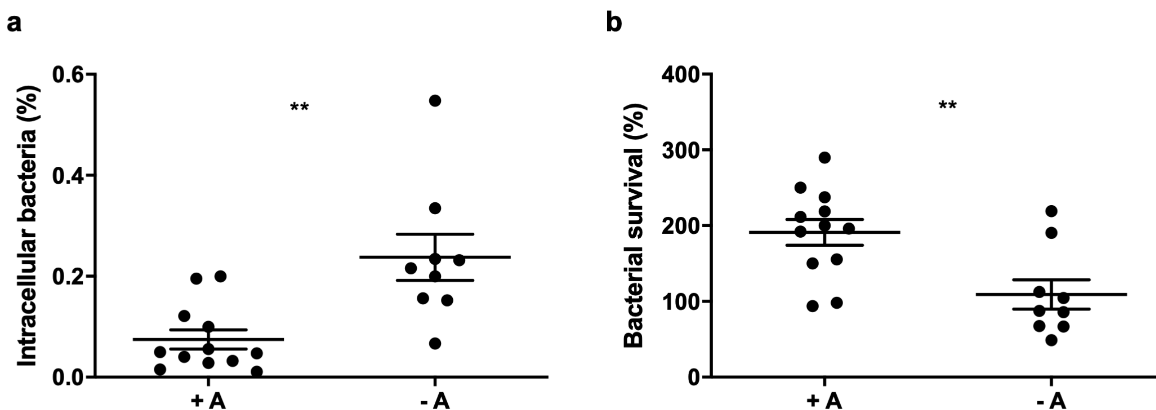
249 (a) amoeba and (b) macrophage cells. Data are presented as Means ± SEM. *p < 0.05; *** p <
250 0.001. (c) Propidium iodide staining of raw 264.7 macrophage cells 24 h after infection with wild
251 type, day 42 amoeba-adapted (A+) and non-adapted (-A) *P. aeruginosa*. Images are shown with
252 and without the fluorescence to better illustrate changes in cell morphology.

253 **Amoeba-adapted *P. aeruginosa* isolates exhibit reduced uptake and enhanced survival in**
254 **the presence of neutrophils**

255 In order to determine if the intracellular survival of amoeba –adapted cells extends to other
256 phagocytic cell types, we compared the ability of adapted and non-adapted *P. aeruginosa* to
257 survive in the presence of human neutrophils. The internalization of 42 d amoeba-adapted and
258 non-adapted populations were determined using a modified gentamicin protection assay.
259 Intracellular CFUs 2 h after infection of non-adapted isolates within neutrophils were higher than
260 amoeba-adapted CFUs (Fig 8a). Amoeba-adapted strains also had increased survival against
261 human neutrophils when compared to non-adapted isolates (Fig 8b).



262

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263 **Fig 8.** Amoeba-adapted *P. aeruginosa* exhibits reduced uptake by and enhanced survival in the
264 presence of human neutrophils. Survival of day 42 day (+ A) amoeba-adapted (4) and (- A) non-
265 adapted (3) strains following incubation with human neutrophils where bacterial (a) uptake and
266 (b) survival were determined. Counts were performed in triplicate and results are the pooled
267 means ± SEM from individual experiments using 3 different donors. Groups were analysed via
268 student T test. **p<0.01

269 ***P. aeruginosa* populations co-incubated with *A. castellanii* show reduced virulence**

270 Most of the phenotypes explored above play a role in the pathogenesis of *P. aeruginosa.*
271 Therefore, we tested for virulence in *Caenorhabditis elegans* fast and slow kill assays. The
272 isolates derived from amoeba-adapted populations after 3 d co-incubation were significantly
273 more toxic to nematodes compared to non-adapted populations, although nematodes exposed to
274 isolates from both populations had a median survival of 8 h (Fig 9a; (χ^2 (1, n = 54) = 31.77, p <
275 0.001). *C. elegans* feeding on isolates from the 42 d non-adapted populations survived longer
276 than when feeding on isolates from the day 3 populations. Furthermore, isolates from the 42 d
277 amoeba-adapted populations were significantly less toxic to *C. elegans* compared to their non-
278 adapted counterparts in both fast kill (Fig 9b; χ^2 (1, n = 54) = 140.7, p < 0.0001) and slow kill
279 assays (Fig 9c; χ^2 (1, n = 54) = 23.52, p < 0.0001).

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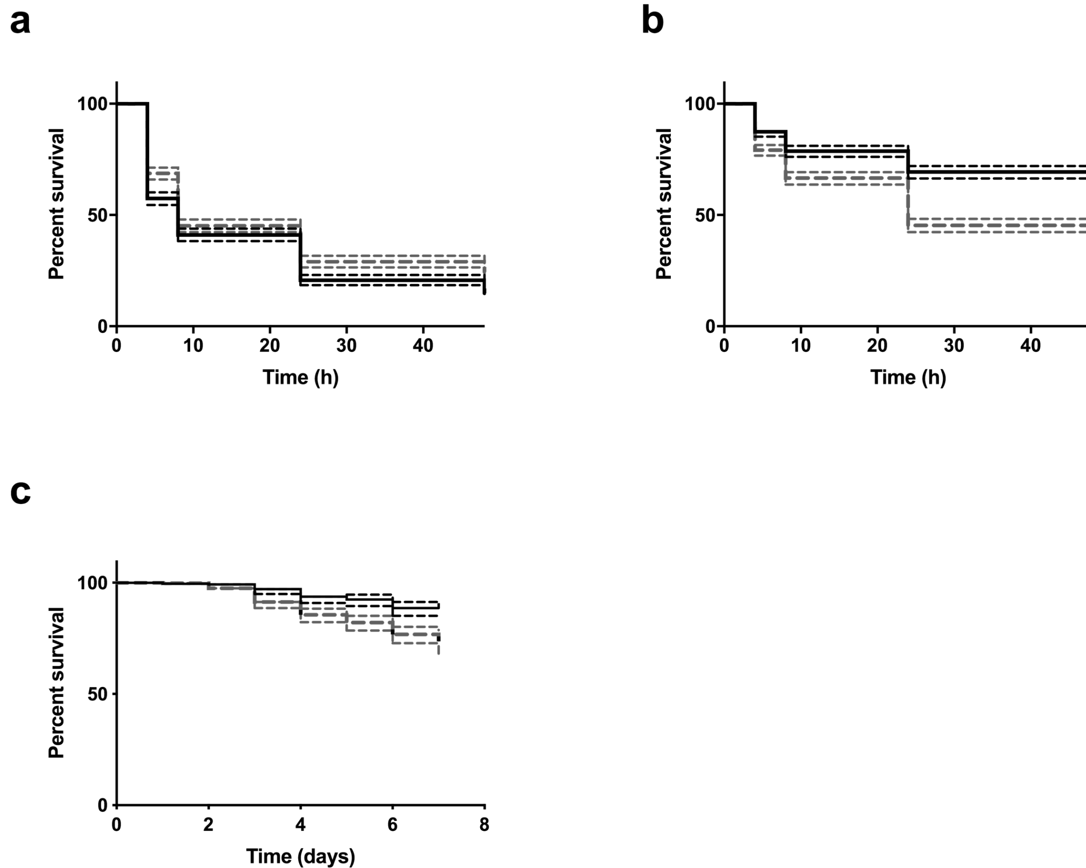

280

281 **Fig. 9. Effect of co-incubation of *P. aeruginosa* with *A. castellanii* in the *C. elegans*

282 virulence assay.** *C. elegans* survival curves after exposure to *P. aeruginosa* isolates derived

283 from amoeba-adapted (black solid bars) or non-adapted (grey dashed bars) populations taken

284 after 3 (a) or 42 (b) d for 0, 4, 8, 24 and 48 h in a fast kill assay. (c) Percent survival of *C.*

285 *elegans* exposed to day 42 isolates of amoeba adapted and non-adapted *P. aeruginosa*

286 populations over 7 days in a slow kill assay. Dotted lines indicate 95% CI.

287 **Discussion**

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288 Most opportunistic pathogens are not transmitted person to person but rather transit through the
289 environment between hosts and therefore, it is likely that the environment plays a significant role
290 in evolution of protective traits. Predation by protists is one of the major mortality factors for
291 bacteria in the environment and it is likely that traits that protect against predation may also
292 impact human hosts during infection (1-3). *P. aeruginosa* is responsible for a variety of
293 nosocomial acute as well as chronic infections (15) in particular chronic lung infections in CF
294 patients (16). Here we investigated the phenotypic and genotypic changes that occur during co-
295 incubation of *P. aeruginosa* with *A. castellanii* for 42 days with a focus on virulence traits.
296 Populations were collected on days 3, 24 and 42 and individual isolates were randomly collected
297 at each time point.

298 **Motility, biofilm formation and secretion of secondary metabolites**

299 Sequencing of populations obtained from the day 42 co-incubation revealed 54 and 65 nsSNPs in
300 adapted and non-adapted populations, respectively. Gene functions of nsSNPs in coenzyme
301 metabolism, lipid metabolism, cell motility and secondary metabolite production occurred solely
302 within the amoeba-adapted population, while COGs representing inorganic ion transport and
303 energy production were over-represented in the non-adapted population (Fig 1). Phenotypic
304 assays of random isolates from the adapted and non-adapted populations confirmed loss of
305 function in many traits associated with these genes. For example, amoeba-adapted isolates
306 showed reductions in twitching and swimming motility from days 3 to 42 and a decrease in
307 swarming motility for day 3 and 24 isolates, but not for the day 42 isolates (Fig 2). In addition,
308 there was a decrease in planktonic growth rate and biofilm formation by amoeba-adapted isolates

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309 compared to non-adapted isolates (Fig 3). It is possible that the loss of biofilm is a result of
310 selection against flagella or adhesive pili as these surface structures are immunogenic and act as
311 ligands for phagocytes, including amoeba (21, 22). In fact, a large number of *P. aeruginosa*
312 isolates from CF patients also lack flagella and pili and these mutants are resistant to
313 phagocytosis by macrophage (23).

314 There were also a number of nsSNPs in genes relating to secretion of secondary metabolites in
315 the adapted population. Phenotypic assays revealed that pyoverdine secretion was significantly
316 reduced in the adapted isolates as early as day 3 and remained low throughout the later time
317 points (Fig 4). Loss of pyoverdine in *P. aeruginosa* in chronic cystic fibrosis infections has also
318 been reported (24). In addition, we observed an overall decrease in production of rhamnolipids,
319 however, this was only a subset of the isolates tested. Unlike other traits that were largely or
320 completely lost, decreases in rhamnolipid secretion were variable, implying that there may be a
321 weaker selective pressure against this phenotype during co-adaptation.

322 **Uptake by and survival in amoeba and immune phagocytes**

323 Results presented here reveal that amoeba-adapted isolates are taken up less readily and have
324 increased intracellular survival in *A. castellanii* and RAW264.7 macrophage cells compared to
325 non-adapted isolates, although the exact mechanisms are not known (Fig 5 and 7). Similarly, we
326 saw reduced internalization of amoeba-adapted isolates by human neutrophils (Fig 8). The loss
327 of immunogenic flagella and pili may contribute to reduced uptake (25), as well as a reduction in
328 chemotaxis due to mutations in chemotaxis genes in all amoeba-adapted populations.

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329 Protozoa have been reported to release dissolved free amino acids and other nutrients when
330 grazing on bacterial prey (26). A reduction in chemotaxis would decrease the encounter of
331 predator and prey since normally *P. aeruginosa* is attracted to amoeba where it attaches to the
332 surface of the amoeba. Likewise changes in LPS also contribute to host avoidance, enhancing
333 fitness of amoeba-adapted strains in the presence of amoeba or macrophage. Interestingly,
334 PA3349 encoding a protein necessary for flagella-mediated chemotaxis was mutated in all
335 amoeba-adapted lineages and is needed for acute but not for chronic *P. aeruginosa* infections
336 (27, 28).

337 The finding that fitness gains from adapting to amoeba could be similarly conferred to
338 interactions with macrophage cells, perhaps demonstrates the overlaps in traits used by *P.*
339 *aeruginosa* to interact with both host types. Much work has been done to show that specialist
340 intracellular pathogens such as *Legionella pneumophila* can evade the defenses of both amoeba
341 and macrophage cells during endocytosis in order to form an intracellular replication niche (29).
342 *P. aeruginosa* is more of an environmental generalist than an intracellular pathogen and has not
343 been observed to form intracellular replication vacuoles, even though it has been recovered from
344 within environmental amoeba (30). In addition, intracellular replication has been described in
345 non-phagocytic epithelial cells and is dependent on a functional type-3 secretion system (31). In
346 the amoeba co-adapted isolates, there was a general decrease rather than increase in intracellular
347 cell numbers, which indicates that it is unlikely that *P. aeruginosa* is replicating intracellularly
348 under the conditions used here.

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349 This work also highlights the complexities of host-pathogen interactions and potential overlaps
350 in these processes, even between cells as far diverged as mammalian macrophage cells and
351 single-celled amoeba. The *A. castellanii* genome contains homologues of interferon-γ-inducible
352 lysosomal thiol reductase enzyme (GILT), interferon inducible GTPase, and the NRAMP
353 homologue, all of which play a role in antimicrobial defense in mammalian cells (32). More
354 research is needed to determine whether these defenses play a role in *P. aeruginosa* infection and
355 whether *P. aeruginosa* possesses mechanisms to evade such defenses in order to survive
356 intracellularly within both phagocytic cell types.

357 ***P. aeruginosa* populations co-incubated with *A. castellanii* show reduced virulence**

358 In order to investigate how constant predation pressure affects *P. aeruginosa* phenotypic and
359 genotypic traits, we adapted *P. aeruginosa* to amoeba for 42 days. Interestingly, the amoeba-
360 adapted isolates were less virulent in both fast and slow-kill *C. elegans* assays (Fig 9). Although
361 the initial 3-day virulence levels were comparable, co-adaptation with amoeba for 42 days
362 resulted in a loss of virulence against *C. elegans* by both fast kill and slow kill mechanisms. Fast
363 and slow killing involves different virulence factors. For example, fast killing is usually due to
364 the production and secretion of diffusible secondary metabolites, while slow killing occurs after
365 colonization and infection of the gut (33). The production of hydrogen cyanide is the main
366 virulence factor involved in the fast killing of *C. elegans* (34), while the regulated export of
367 proteins is needed for slow killing (33).

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368 Consistent with the reduction in *C. elegans* killing, we observed reductions in key virulence
369 traits, such as motility, biofilm formation, and secondary metabolite production in days 24 and
370 42 amoeba-adapted isolates. The loss of motility and secondary metabolites probably
371 contributes directly to reduced *C. elegans* mortality. In *P. aeruginosa*, type-IV pili are involved
372 in adherence, swarming and twitching motility, and virulence. *P. aeruginosa* mutants deficient
373 in *pilA* and *pilT* demonstrate reduced pathogenesis in mice compared to the wild type (35).
374 Virulence towards nematodes has been partly attributed to pyoverdine. For example, when
375 *Pseudomonas syringae* interacted with *C. elegans*, the genes, *pvdJ* and *pvdE* that are involved in
376 the synthesis of pyoverdine, were significantly upregulated on fast kill agar (36). A similar
377 response was observed in *P. aeruginosa*, where 'red death' type killing of *C. elegans* was shown
378 to be partly due to the production of pyoverdine (37). Additionally, *P. syringae* Δ*pvdJ* and
379 Δ*pvdL* mutants were unable to produce pyoverdine and an unrelated toxin, tabtoxin,
380 demonstrated reduced AHL production and attenuated virulence against the tobacco plant host
381 (38).

382 Rhamnolipids are quorum-sensing regulated secondary metabolites expressed in biofilms when
383 neutrophils are present (39) that lyse macrophages, neutrophils and protozoans. If the gene is not
384 expressed under the conditions of our co-adaptation, it may not be under strong negative
385 selective pressures. Regulation of expression may be one mechanism that allows for the retention
386 of certain virulence traits under predation.

387 **Commensalism as an adaptive strategy**

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388 The co-adapted isolates had mutations in many virulence phenotypes, including loss of motility,
389 slower growth and increased intracellular survival. These adaptations represent a commensal
390 strategy that may indicate adaptation towards co-existence with amoeba. *P. aeruginosa*
391 consistently adopts a more commensal lifestyle during adaptation to different hosts. For
392 example, experimental evolution experiments with *C. elegans* demonstrated that *P. aeruginosa*
393 evolved an attenuated virulence phenotype after serial passages (12). This is in contrast to other
394 pathogens where virulence has been demonstrated to increase after serial passages (40). The
395 decrease in acute virulence phenotypes also occurs in *P. aeruginosa* strains isolated from
396 chronically infected CF patients (17, 41, 42).

397 In co-evolution with *C. elegans*, *lasR* and *rhlR* quorum sensing mutations occurred early in the
398 adaptation process. In addition, *lasR* mutations are prevalent across multiple CF isolates (43).
399 The regulatory genes *lasR* and *rhlR* control the expression of many virulence genes, and *las* and
400 *rhl* mutants have been shown to be less virulent in models of wound infection (44). It has been
401 proposed that pleiotropic adaptive mutations in global regulatory genes are more likely to occur
402 than multiple mutations in individual virulence traits. Mutations in *lasR* occurred in one of the
403 three amoeba-adapted replicates, so stronger selection may be driving the loss of individual
404 virulence traits in the other amoeba-adapted populations. In CF lineages, *P. aeruginosa*
405 adaptations include gains in mucoidy and antibiotic resistance, and loss of secondary metabolites
406 and motility (17), of which the latter two were observed in the amoeba-adapted populations. The
407 parallel losses of secondary metabolites and motility are striking and evoke the question of
408 whether the selective forces driving these traits are the same in both systems.

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409 Advantageous traits may be lost if the cost of maintaining the traits outweigh the benefits. Loss
410 of pyoverdine in *P. aeruginosa* CF infections has been shown to be driven by social selection
411 (45). Pyoverdine is a strong scavenger for iron and non-producing cheats retain the pyoverdine
412 receptor and are able to uptake iron. Only when extrinsic pyoverdine is completely lost do
413 mutations appear in the receptor genes. In our study, although there were many *pvd* mutations,
414 we did not observe any mutations in the receptor genes, suggesting that social selection may also
415 be at play here. It is also possible that other avenues of iron uptake are preferentially utilized
416 (46), as iron uptake occurs via *hemO* in late CF strains (47).

417 Strong negative selection against traits could also occur due to host recognition and the need for
418 evasion by pathogens. Flagellin is the site of recognition by mammalian toll-like receptors,
419 resulting in immune activation (22), and the site where *A. castellanii* binds *P. aeruginosa* before
420 it is endocytosed (21). Additionally, flagellin and TLR-independent loss of motility has been
421 shown to significantly reduce phagocytic uptake by mammalian immune cells (48), and may
422 explain why loss of motility is such a strong selection factor in the co-adaption study presented
423 here and in CF lineages. Several findings presented here support predator avoidance as a
424 selection pressure. The parental *P. aeruginosa* strain exhibits chemotaxis and rapidly swims
425 towards and are taken up by amoeba and macrophage cells within seconds of exposure.
426 However, amoeba-adapted isolates do not attach to the surface of the amoeba. This is supported
427 by experiments showing reduced uptake of amoeba-adapted populations by amoeba and
428 macrophage. Loss of flagella and motility is therefore adaptive for the purpose of predator
429 avoidance. Additionally, many chemotaxis mutants were detected in the population genomic
430 data. For example, *pctB* and PA3349 were mutated in all three independent experimental

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431 amoeba-adapted populations. *pctB* is a methyl-accepting chemotaxis protein with a high affinity
432 to glutamine (49), which has been shown to be involved in chemotaxis to wounded airway
433 epithelial cells (50). The loss of chemotaxis in the amoeba-adapted strains would be consistent
434 with predator avoidance.

435 In this study, it has been demonstrated for the first time that adaptation to a more commensal
436 lifestyle may also confer benefits in an infectious context for a generalist pathogen, for it is clear
437 that although amoeba-adapted cells are less virulent, they are still capable of invading and
438 colonizing the *C. elegans* model. Similarly, adapted CF strains have been shown to be equally as
439 capable as environmental strains of infecting a new host in a mouse model (51). Although
440 amoeba may be thought of as training grounds for the formation of virulence traits, they may
441 also be grounds for a more 'chronic' co-existence.

442 Materials and methods

443 Organisms and growth conditions

444 *P. aeruginosa* strain DK1 used for this study was initially obtained from a Danish CF patient
445 (P30M0) (18). Unless otherwise stated, *P. aeruginosa* DK1 strain and population-derived
446 isolates were grown in 10 mL lysogeny broth (LB_{10}, BD Biosciences, USA) at 37 °C with
447 shaking at 200 rpm. *A. castellanii* was obtained from the American Type Culture Collection
448 (ATCC 30234) and was routinely maintained axenically in peptone-yeast-glucose (PYG)
449 medium (20 g protease peptone, 5 g yeast extract, and 50 mL 2 M glucose L^{-1}) at room

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450 temperature. Prior to use in experiments, *A. castellanii* was passaged and washed twice with 1 ×
451 phosphate buffered saline (PBS; Sigma-Aldrich, USA) solution to remove PYG media. *C.*
452 *elegans* N2 Bristol was maintained on nematode growth medium (NGM) (per liter; 2.5 g Bacto-
453 Peptone (BD Biosciences, USA), 3 g NaCl, 7.5 g agar, 1 mL 5 mg mL^{-1} cholesterol, 1 mL 1 M
454 $MgSO_4$, 1 M $CaCl_2$, and 25 mL 1 M potassium phosphate buffer at pH 6) fed with *E. coli* OP50.

455 RAW 264.7 macrophage cell lines (ATCC TIB-71) were grown in Dulbecco's modified Eagle
456 Medium (DMEM; Thermo Fisher, USA) with 10 % fetal bovine serum (FBS) at 37 °C with 5 %
457 CO_2. Before use, cells were washed with PBS and treated with trypsin briefly before gentle
458 detachment by scraping. Cells were then centrifuged at 1000 × *g* for 1 min and resuspended in
459 experimental media before use.

460 ***P. aeruginosa* and *A. castellanii* co-incubation**

461 Overnight cultures of *P. aeruginosa* grown in was centrifuged at 4000 × *g* for 5 min and washed
462 twice with 1 × M9 salts solution (Sigma-Aldrich, USA; per litre, 6.78 g Na_2HPO_4, 3 g H_2PO_4, 1 g
463 NH_4Cl, 0.5 g NaCl). *A. castellanii,* at a concentration of 1×10^3 cells mL^{-1}, was seeded onto the
464 surface of 25 cm^2 tissue culture flasks with 0.2 µm vented caps filled with 10 mL 1 × complete
465 M9 salts + 0.01% glucose. To maintain a strong selective pressure from amoeba, 100 µL of *A.*
466 *castellanii* and *P. aeruginosa* was taken from percussed, 3-day-old established flasks and added
467 to new flasks containing *A. castellanii* every 3 d. Three independent populations of *P.*
468 *aeruginosa* with *A. castellanii* were established (amoeba-adapted populations).

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469 In parallel with the co-incubation experiment, *P. aeruginosa* were maintained without *A.*
470 *castellanii*. Briefly, *P. aeruginosa* was diluted to a cell concentration of 1 $\times 10^2$ cells mL^{-1} and
471 added to tissue culture flasks containing 10 mL 1 × complete M9 + 0.01 % glucose. From these
472 flasks, 100 µL of 3-day-old established *P. aeruginosa* culture was added to flasks containing
473 fresh media every 3 d. Three independent populations of *P. aeruginosa* were established (– A
474 populations).

475 Prior to re-inoculation and sampling, the biomass of each population was measured by
476 spectrophotometry (Infinite® M200, Tecan, Switzerland) at 600 nm. The number of *A.*
477 *castellanii* within each amoeba-adapted population were enumerated using inverted microscopy
478 (Zeiss Axio Observer.Z1 inverted wide-field).

479 **Isolation of intracellular *P. aeruginosa* populations**

480 On days 3, 24 and 42, flasks containing *A. castellanii* (amoeba-adapted) were percussed until the
481 amoebae detached and 1mL of the culture media was filtered through a 3 µm cellulose acetate
482 membrane (Merck, Germany) to retain the *A. castellanii*. *A. castellanii* were resuspended in 5
483 mL of 1 × M9 salts and pelleted at 4000 × g for 5 min before resuspension in 100 µL 1 × M9
484 salts solution. *A. castellanii* were lysed by the addition of 100 µL of 1 % Triton –X for 1 min,
485 the mixture was then pelleted and washed twice with 900 µL of 1 × M9 salts. The cell pellet was
486 resuspended in 1 mL of 70 % LB_{10} + 30 % glycerol and stored at -80 °C. The same treatment
487 was applied to the *P. aeruginosa* cells from the non-adapted populations.

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488 **Sequencing of *P. aeruginosa* populations and computational tools**

489 *P. aeruginosa* populations were sequenced to determine genotypic changes that occurred in
490 response to co-adaptation with amoeba. Genomic DNA was extracted from the parental wild
491 type strain, and each amoeba-adapted and non-adapted population derived from days 3 and 42
492 using the QIAamp DNA mini kit (Qiagen, Venlo, Netherlands) according to manufacturer's
493 instructions. Sequencing libraries were prepared using the TruSeq DNA sample preparation kit
494 (Illumina, San Diego, CA, USA), and sequenced on a MiSeq (Illumina, USA).

495 Reads were aligned to the *P. aeruginosa* PAO1 reference genome using CLC Genomics
496 Workbench 9 (CLC Bio, Aarhus, Denmark). SNPs and small insertions and deletions (indels)
497 were called using the probabilistic variant detection analysis, and mutations that were also in the
498 parental strain were filtered out. Genes that contained SNPs in > 25% of the total gene reads
499 were selected for functional analysis using the Database for Annotation, Visualization and
500 Integrated Discovery (DAVID) v6.8 functional tools (52, 53). Gene lists were uploaded onto the
501 DAVID website (https://david.ncifcrf.gov/) and annotations were limited to *P. aeruginosa*. A
502 functional annotation table was compiled for amoeba adapted populations obtained from day 42
503 of the experiment (Table 1).

504 **Isolation of adapted strains**

505 To facilitate further phenotypic analysis, *P. aeruginosa* cells obtained from amoeba-adapted and
506 non-adapted populations on days 3, 24 and 42 were plated onto LB_{10} agar and incubated

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507 overnight at 37°C. Ten colonies were randomly selected from each population using a numbered
508 grid and a random number generator. These isolates were stored in 1 mL 70 % LB_{10} + 30 %
509 glycerol at -80 °C.

510 **Assessment of surface colonization and planktonic growth**

511 To determine if adaptation with *A. castellanii* altered surface colonization by *P. aeruginosa*, the
512 biomass of attached cells was quantified by crystal violet staining as previously described (54).
513 Briefly, overnight cultures of each isolate were added to 96 well plates containing 100 µL 1 ×
514 M9 + 0.4 % glucose. Plates were incubated at room temperature with agitation at 80 rpm for 24
515 h. To separate the suspended biomass from the attached biomass, each well was washed once
516 with 1 × PBS. Cells attached to the plate surface were stained with 200 µL of 0.3 % crystal
517 violet and incubated for 20 min, after which unbound crystal violet was removed by washing 3
518 times with 1 × PBS. Crystal violet was liberated from the cells with 300 µL of 10 mL of
519 absolute ethanol. The absorbance of the crystal violet was measured with a spectrophotometer at
520 590 nm (Infinite® M200, Tecan, Switzerland) in triplicate.

521 The planktonic growth rate of each isolate was assessed by adding log phase bacterial cultures to
522 96 well plates containing 200 µL of 1 × M9 + 0.01 % glucose and incubating with agitation at 80
523 rpm for 18 h at 37ºC. The suspended biomass within each well was transferred to a fresh plate
524 and measured by spectrophotometry ($OD_{600\ nm}$) (Infinite® M200, Tecan, Switzerland). The
525 specific growth rate was determined by applying the formula µ = 2.303 ((log OD2) – (log

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526 OD1)/(t2-t1)). Assessment of the planktonic growth rate was performed in triplicate for each
527 isolate, n = 30 for each time and treatment.

528 **Motility assays**

529 Twitching, swarming and swimming motility were assessed as previously described, using
530 motility agar (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl,1 mM $MgSO_4$,
531 100 µM $CaCl_2$, 2 gL^{-1}Dextrose, 5 g L^{-1}casamino acids) containing 1, 0.5 or 0.3 % wt vol^{-1}
532 agarose (Bacto™ , BD Biosciences, USA), respectively (55, 56). Five milliliters of motility agar
533 were added into the wells of 6 well plates and dried under laminar flow for 1 h. Isolates were
534 inoculated into the center of the well using 10 µL pipette tips, either to the base of the plate for
535 assessment of twitching motility or mid-agar for assessment of swimming and swarming.
536 Twitching and swarming plates were incubated at room temperature for 48 h and swimming
537 plates were incubated for 24 h prior to imaging with a digital camera (Canon EOS 600D digital
538 single-lens reflex (DSLR) mounted on a tripod, to allow for phenotypic characterization of the
539 resulting colonies and comparative endpoint twitch, swarm and swim distances. Determination
540 of the zone of motility was semi-quantitatively analyzed using ImageJ image analysis software.
541 Motility was assessed in triplicate (n = 3).

542 **Quantification of pyoverdine**

543 To determine if adaptation with amoeba (3, 24 and 42 d) affects the production of pyoverdine,
544 isolates were grown overnight in 1 mL LB_{10} media. Cells were removed by centrifugation at

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545 5200 × *g* for 5 min and the absorbance of the supernatant was determined with a
546 spectrophotometer (Infinite® M200, Tecan, Switzerland) at excitation 400 nm and emission 460
547 nm in triplicate.

548 **Quantification of rhamnolipids**

549 The orcinol method (57) was used to quantify the production of rhamnolipid biosurfactant of
550 nine randomly selected adapted and non-adapted isolates from the day 42 population. Briefly,
551 overnight *P. aeruginosa* LB cultures were diluted to OD_{600} 0.01 in 25 mL of AB minimal media
552 (58) supplemented with 2 g glucose and 2 g casamino acids L^{-1} and grown overnight at 37 °C
553 with shaking at 200 rpm. The cell density was determined ($OD_{600\ nm}$) before filtration and
554 extraction of crude rhamnolipid from the supernatant two times using diethyl ether (7 mL). The
555 organic layer was collected, combined, and concentrated in a vacuum concentrator (SpeedVac,
556 Thermo Scientific) at 0 °C for 1 h followed by 2 h at 25 °C, until white solids formed. The solids
557 were resuspended in 500 µL of water and 50 µL of this solution was mixed with 450 µL of
558 freshly prepared orcinol (0.19 % in 53 % H_2SO_4). Samples were incubated at 80 °C for 30 min
559 and allowed to cool at room temperature for 15 min before quantification of absorbance (OD_{421}).
560 The absorbance was normalized to cell concentration ($OD_{600\ nm}$) for each sample and a factor of
561 2.5 was applied to convert values from a rhamnose standard curve to rhamnolipid concentration
562 (59).

563 **Nematode survival assay**

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564 To determine if *P. aeruginosa* adaptation to *A. castellanii* altered bacterial virulence, we tested
565 the survival of *C. elegans* sp. Bristol N2 after feeding on *P. aeruginosa*. Axenic *C. elegans* were
566 obtained via the egg-bleach synchronization method, plated onto NGM agar and fed with heat-
567 killed *Escherichia coli* OP50. L4 stage worms were re-suspended in 1× M9 salts solution and 10
568 - 30 worms were drop plated onto 35 mm dishes containing 2 mL fast or slow kill agar (33)
569 containing lawns of pre-established *P. aeruginosa* obtained from amoeba-adapted or non-
570 adapted populations. Plates were incubated at 22 °C and worm numbers were scored by
571 microscopy at 0, 4, 8, 24 and 48 h for fast kill assays, and once per day for slow kill assays.
572 Nematode toxicity was tested using 9 randomly selected *P. aeruginosa* isolates from each
573 treatment and from times 3 and 42 d. Nematode survival assays were repeated twice
574 independently, and each experiment was performed in triplicate.

575 **Fluorescent tagging of isolates**

576 To prepare fluorescently-tagged strains of *P. aeruginosa* obtained from amoeba-adapted and
577 non-adapted populations, two isolates were randomly selected from the 42 d population and
578 grown overnight at 37 °C in LB broth. Electroporation was performed as previously described
579 (60). One milliliter of *P. aeruginosa* at a cell density of 1×10^8 cells mL^{-1} was pelleted and
580 washed twice with 300 mM sucrose. The expression tag carrying plasmid pUC18-TR6K-mini-
581 Tn7T-Gm-GFP (0.5 µg) (expresses a green fluorescent protein *gfp*; emission 488 nm/excitation
582 509 nm) (61) or pUC18T-miniTn7T-Gm-Mcherry (0.5 µg) (expresses a red fluorescent protein
583 mCherry emission 587 nm/excitation 610 nm) (62) was mixed with 1 µg pTNS1 helper plasmid
584 and 300 µl resuspended bacteria, in a 2-mm-gap electroporation cuvette. This mixture was

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585 electroporated at 1.8kV/25μF/2100 Ω, 2.5kV cm^{-1} in a Gene Pulser apparatus (BIO-RAD,
586 Hercules, CA, USA). Cell recovery was performed in ice-cold super optimal broth with
587 catabolite repression media (SOC) (10 mM NaCl, 2.5m M KCl, 10m M $MgCl_2$, 10m M $MgSO_4$,
588 20 g L^{-1} tryptone, 5 g L^{-1} yeast extract + 2% glucose. Cells were incubated with shaking for 3h
589 at 37 ºC. One hundred microliters of culture were plated onto LB_{10} plates supplemented with
590 200 ug mL^{-1} gentamicin to select for GFP and mCherry transformants. Bacterial stocks were
591 derived from a single transformed colony.

592 **Competition assays**

593 To determine if prior exposure of *P. aeruginosa* to amoeba increased competitiveness when
594 grown with amoeba, a competition assay was performed. One isolate from the 42 d amoeba
595 adapted population and one isolate from the 42 d – A population were fluorescently tagged as
596 previously described. *A. castellanii*, at a cell concentration of 1×10^5 cells mL^{-1} was added to 24
597 well plates (Falcon) in 450 µL 1 × M9 salts + 0.01 % glucose solution. Overnight cultures of
598 GFP or mCherry-labelled *P. aeruginosa* were grown in LB_{10} broth supplemented with 200 µg
599 gentamicin at 37 °C with agitation at 200 rpm. The amoeba adapted::gfp and – A::mCherry
600 isolate or the amoeba adapted ::mCherry and the – A::gfp isolate were mixed in equal proportion
601 and added to the wells containing amoeba to a final bacterial cell concentration of 2×10^6 cells
602 mL^{-1}. Each experiment was conducted in triplicate. The plates were incubated at room
603 temperature with agitation at 60 rpm for 48 h before imaging on a Zeiss Z1 inverted wide field
604 microscope. Acquired images were deconvoluted in Autoquant X3 (Bitplane) before

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605 quantification of the relative red and green fluorescence in the field of view using Imaris 8
606 (Bitplane).

607 **Growth of *P. aeruginosa* populations in amoeba supernatant**

608 To investigate whether fitness differences were due to enhanced growth by utilizing resources
609 released by amoeba, nine randomly selected day 42 adapted and non-adapted *P. aeruginosa*
610 isolates were grown in media supplemented with 50 % amoeba supernatant in order to compare
611 their growth rates. Amoeba supernatant was obtained by growing *A. castellanii* in M9 salts
612 minimal media + 0.04 % glucose with heat killed *P. aeruginosa* (made by incubating overnight
613 cultures at 65 ^{o}C for 3 h and plating on LB agar to check that no live cells remain) for 3 d, and
614 then filtering the suspension through an 0.22 µm filter (Pall, USA). Overnight cultures of *P.*
615 *aeruginosa* isolates grown in LB were adjusted to the same optical density ($OD_{600\ nm}$) and added
616 to amoeba supernatant or M9 salts + 0.04 % glucose. Planktonic growth rates were quantified
617 from optical density readings ($OD_{600\ nm}$) using a Tecan microplate reader as previously
618 described.

619 **Uptake and intracellular survival of *P. aeruginosa* populations in macrophages**

620 To investigate the dynamics of uptake and intracellular survival of 42 d +A and -A *P. aeruginosa*
621 populations within macrophages, overnight LB cultures of adapted and non-adapted *P.*
622 *aeruginosa* populations were added to RAW264.7 macrophage cells (5×10^4 cells/well in 96-
623 well tissue culture plates) in DMEM without FBS at a multiplicity of infection (MOI) of 100:1.

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624 The infected cells were incubated at 37 ^{o}C with 5 % CO_2. After co-incubation for 1 h, the media
625 was removed and replaced with media containing 100 μg mL^{-1} gentamicin to kill extracellular
626 bacteria. Macrophage were washed with PBS and lysed at 3, 5, 7, 12, and 18h post-infection and
627 CFU counts were performed to enumerate surviving intracellular cells. Propidium iodide
628 (ThermoFisher LIVE/DEAD Cell Viability kit) staining was done to determine the state of the
629 host cells 24h post-infection.

630 **Uptake and survival of *P. aeruginosa* populations in the presence of neutrophils**

631 *P. aeruginosa* (4 adapted and 3 non-adapted) strains from overnight culture were washed once in
632 PBS then diluted in PBS (OD=0.1, ~$1x10^8$) and resuspended in complete media (RPMI + 2%
633 heat inactivated autologous plasma) to experimental concentrations just prior to infection.
634 Neutrophils were isolated from whole blood, collected from healthy donors in lithium heparin
635 vacutainer tubes and separated using polymorphprep (axis shield) and centrifugation. RBCs were
636 hypotonically lysed and neutrophils washed in HBSS (without Ca^+ or Mg^+). Neutrophils were
637 counted and resuspended at their final concentration in complete media. In a 96-well plate,
638 neutrophils were added to wells for challenge (neutrophil+) and complete media added to control
639 wells (neutrophil-). *P. aeruginosa* was added to both PMN+ and PMN- wells at a MOI of 100:1
640 and incubated for 1 h at 37°C, 5% CO2. After co-incubation for 1 h bacterial survival was
641 determined by serial dilution and plating on LB for enumeration. Uptake was determined by
642 media removal and replacement with media containing 100 μg mL^{-1} gentamicin to kill
643 extracellular bacteria. At the experiment endpoint a sample of infection was taken and lysed in a
644 new 96 well plate, followed by serial dilution and plating onto LB agar. CFUs were determined

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645 by counting and percent inoculum determined as [CFU of neutrophil + wells/CFU neutrophil-
646 wells x 100]. Counts were performed in triplicate and results are the pooled Means ± SEM from
647 individual experiments using 3 different donors.

648 **Human Ethics**

649 Ethics for whole blood collection was obtained from the University of Wollongong Human
650 research Ethics Committee (HREC # 08/250).

651 **Statistical analyses**

652 Phenotypic differences between *P. aeruginosa* isolates obtained from amoeba-adapted and non-
653 adapted populations at specific time points (3, 24 and 42 d) were determined by ANOVA, with
654 amoeba adaptation (with or without *A. castellanii*) as a fixed factor and adaptation time (3, 24,
655 42 d) as a random factor. Multiple testing was conducted using the Tukey Post-hoc Test. All
656 phenotypic data were log transformed (ln (x+1)) prior to analysis to improve normality. *P*-
657 values < 0.05 were considered significant. Nematode survival curves were constructed with
658 GraphPad Prism v 6.0 using the Kaplan-Meier method. Differences between nematode survival
659 after exposure to *P. aeruginosa* isolates from amoeba- adapted or non-adapted populations were
660 determined using log-rank tests with significance given to *p*-values < 0.05. Differences between
661 neutrophil uptake and survival of amoeba-adapted and non-adapted strains were analyzed via
662 student's T tests.

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663 **Table 1** Non-synonymous SNPs and gene deletions in genes associated with motility and
664 secondary metabolism that occurred solely in amoeba-adapted 42 d populations

Gene	Functional annotation	Frequency % (population #)	Locus: SNP [(a)]	Amino acid substitution [(b)]
Motility				
flgF/ PA1081	Flagellar basal-body rod	55.17 (1)	NP_249772.1: c.562G>T	p.Glu188*
flgH/PA1083	Flagellar L-ring protein precursor	34.62 (1)	NP_249774.1: 4:c.87G>T	p.Gly163Cys
flgK/PA1086	Flagellar hook-associated protein 1	42.31 (2)	NP_249777.1: c.1182C>G	p.Tyr394*
		96.43 (3)	NP_249777.1: c.794delA	p.Asn266fs
fimV/PA3115	Motility	27.78 (2)	NP_251805.1: c.1219A>C	p.Thr407Pro
ctpL/ PA4844	Chemotaxis	38.46 (1)	NP_253531.1: c.463A>C	p.Thr155Pro
pilM	Type IV fimbrial biogenesis	46.43 (2)	NP_253731.1: c.576_577delGT	p.Gln192fs

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pilN	Type IV fimbrial biogenesis	39.53 (1)	NP_253730.1: c.442G>T	p.Glu148*
pilT	Twitching motility	25.53 (3)	NP_249086.1: c.562_573delGCGCTGCGCTCG	p.Ser191_Arg194del
Secondary metabolism				
yfiH/ PA4543	Multi-copper polyphenol oxidoreductase	29.41 (2)	NP_253233.1: c.77T>G	p.Val26Gly
yafE/PA3119	Methyltransferase activity	38.89 (1)	NP_251809.2: c.401T>G	p.Val134Gly
		25 (3)	NP_251809.2: c.401T>G	p.Val134Gly
pvdD	Pyoverdine synthetase D	30 (1)	NP_251089.1: c.1582T>G	p.Leu528Val
		33.33 (2)	NP_251089.1: c.2141T>G	p.Val714Gly
pvdJ/PA2400	Pyoverdine biosynthetic process	25 (3)	NP_251090.2: c.5665C>G	p.Arg1889Gly

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665 [a]SNPs are indicated by the accession number within their codon context, in the order: nucleotide
666 N in protein coding sequence; codon population non-adapted > codon population amoeba-
667 adapted. c. indicates coding reference sequence; del indicates gene deletion.

668 [b]p. indicates protein reference sequence; Arg = arginine, Asn = asparagine, Cys = cysteine, Gln =
669 glutamine, Glu = glutamic acid, Gly = glycine, Leu = leucine, Ser = serine, Thr = threonine, Tyr
670 = tyrosine, Val = valine.

671 *indicates translation termination (stop) codon; fs indicates frameshift;

672 **Acknowledgments**

673 The authors would like to acknowledge support from The ithree Institute at the University of
674 Technology Sydney, Sydney, Australia, the Australian Research Council Discovery Project
675 (DP170100453) and by the National Research Foundation and Ministry of Education Singapore
676 under its Research Centre of Excellence Program to the Singapore Centre for Environmental Life
677 Sciences Engineering, Nanyang Technological University.

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