1 **Adaptation to an amoeba host leads to *Pseudomonas aeruginosa* isolates**

2 **with attenuated virulence**

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22 ## Abstract

23 The opportunistic pathogen *Pseudomonas aeruginosa,* is ubiquitous in the environment, and
24 in humans is capable of causing acute or chronic infections. In the natural environment,
25 predation by bacterivorous protozoa represents a primary threat to bacteria. Here, we
26 determined the impact of long-term exposure of *P. aeruginosa* to predation pressure. *P.*
27 *aeruginosa* persisted when co-incubated with the bacterivorous *Acanthamoeba castellanii* for
28 extended periods and produced genetic and phenotypic variants. Sequencing of late-stage
29 amoeba-adapted *P. aeruginosa* isolates demonstrated single nucleotide polymorphisms
30 within genes that encode known virulence factors and this correlated with a reduction in
31 expression of virulence traits. Virulence towards the nematode, *Caenorhabditis elegans,* was
32 attenuated in late-stage amoeba-adapted *P. aeruginosa* compared to early-stage amoeba-
33 adapted and non-adapted counterparts. Further, late-stage amoeba-adapted *P. aeruginosa*
34 showed increased competitive fitness and enhanced survival in amoeba as well as in
35 macrophage and neutrophils. Interestingly, our findings indicate that the selection imposed by
36 amoeba resulted in *P. aeruginosa* isolates with reduced virulence and enhanced fitness,
37 similar to those recovered from chronic cystic fibrosis infections. Thus, predation by protozoa
38 and long-term colonization of the human host may represent similar environments that select
39 for similar losses of gene function.

40 ## Importance

41 *Pseudomonas aeruginosa* is an opportunistic pathogen that causes both acute infections in
42 plants and animals, including humans, and chronic infections in immunocompromised and
43 cystic fibrosis patients. This bacterium is commonly found in soils and water where bacteria
44 are constantly under threat of being consumed by bacterial predators, e.g. protozoa. To
45 escape being killed, bacteria have evolved a suite of mechanisms that protect them from
46 being consumed or digested. Here, we examine the effect of long-term predation on the

47 genotypes and phenotypes expressed by *P. aeruginosa*. We show that long term co-
48 incubation with protozoa resulted in mutations that resulted in *P. aeruginosa* becoming less
49 pathogenic. This is particularly interesting as we see similar mutations arise in bacteria
50 associated with chronic infections. Importantly, the genetic and phenotypic traits possessed
51 by late-stage amoeba-adapted *P. aeruginosa* are similar to what is observed for isolates
52 obtained from chronic cystic fibrosis infections. This notable overlap in adaptation to
53 different host types suggests similar selection pressures amongst host cell types as well as
54 similar adaptation strategies.

55 Introduction

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

63 Predation by protists, or protozoa, is a major mortality factor for bacteria in the environment
64 (4). Virulence traits that cause human disease, are hypothesized to have evolved in response
65 to, and are maintained by, predation pressure, which supports the "coincidental evolution"
66 hypothesis. This hypothesis states that virulence is a coincidental consequence of adaptation
67 to other ecological niches (5-7). Coincidental evolution is supported by examples of factors
68 that play roles in both grazing resistance and virulence towards mammalian cells (7-10),
69 including traits such as cell-surface alterations, increased swimming speed, toxin secretion
70 and biofilm formation (5, 7) . Conversely, virulence traits may be attenuated or lost when

71 organisms adapt to form a more commensal relationship with a host (11-14). Microorganisms
72 may also develop specific virulence traits against a specific host becoming a specialist
73 pathogen. Although there are many hypotheses for how virulence traits evolve, there have
74 been few experimental evolution studies on the adaptation of specific virulence traits to
75 different host types and environments (15-18). Such studies are particularly important for
76 understanding how opportunistic pathogens evolve (19).

77 *P. aeruginosa* is a versatile opportunistic pathogen found in a wide variety of natural habitats.
78 *P. aeruginosa* has a large (approximately 6.3 Mb) genome containing many genes for
79 metabolism and antibiotic resistance (20), and coupled with a complex regulatory network,
80 this organism is able to survive in a variety of niches. It is an important human pathogen,
81 responsible for both acute nosocomial infections (21) and chronic infections, e.g. in the lungs
82 of cystic fibrosis (CF) patients (22). In the CF lung, it has been shown to adapt to a more
83 commensal lifestyle by altering the expression of acute virulence traits such as motility,
84 quorum sensing and toxin production (23).

85 While there are many studies addressing the evolution of *P. aeruginosa* in the CF lung (23-
86 25), there is less known about the impact of environmental factors such as protozoan
87 predation on the evolution of virulence. To address this lack of knowledge, here we
88 investigated the adaptation of *P. aeruginosa* during long-term co-incubation with the amoeba,
89 *Acanthamoeba castellanii*. *P. aeruginosa* was co-evolved with *A. castellanii* for 42 days and
90 the impact of co-evolution assessed for a range of phenotypes, including virulence in a
91 *Caenorhabditis elegans* infection model. Adapted populations as well as selected isolates
92 were also sequenced to investigate the range of mutations that occurred during co-incubation.

93 ## Results

94 Here, we examined the effects of long-term co-adaptation of *P. aeruginosa* to the amoeba, *A.*
95 *castellanii*. Triplicate populations of *P. aeruginosa* were co-adapted with and without *A.*
96 *castellanii* for 42 days in M9 minimal medium. Non-adapted control isolates were incubated
97 in M9 medium, while intracellular adapted isolates were collected from amoeba every 3 days.
98 Phenotypic changes of adapted and non-adapted isolates were determined using various
99 assays as indicated in the following sections. To identify the underlying genetic changes
100 associated with these phenotypes, sequencing of day 3 and 42 populations and single isolates
101 were performed.

102 **Effect of long-term adaptation on virulence factor production**

103 To identify alterations in phenotypes expressed by amoeba-adapted and non-adapted isolates,
104 motility, biofilm formation, pyoverdine and rhamnolipid production were assessed. Nine
105 randomly selected individual isolates of *P. aeruginosa* each from adapted and non-adapted
106 population on days 3, 24 and 42 were assessed. The long-term co-incubation of *P. aeruginosa*
107 with amoeba resulted in a reduction in twitching motility ($F_{2, 534} = 295.1$, $p < 0.001$) (Fig
108 1.A). Amoeba-adapted and non-adapted *P. aeruginosa* isolates from day 3 did not differ
109 significantly ($p = 0.53$), however, on days 24 and 42 twitching motility was significantly
110 reduced compared to the non-adapted isolates ($p < 0.001$). The mean twitching motility of
111 amoeba-adapted isolates was 10-fold less than isolates that were incubated in the absence of
112 *A. castellanii* ($p < 0.001$).

113 Co-incubation with *A. castellanii* also resulted in a decrease in swimming motility ($F_{2, 534}$ =
114 15.6, $p < 0.001$), where amoeba-adapted isolates from day 3 showed a swim area half that of
115 isolates from non-adapted isolates ($p < 0.001$) (Fig 1.B). This pattern of reduced swimming
116 motility was also observed for amoeba-adapted isolates from days 24 and 42 ($p < 0.001$ and p

117 < 0.001, respectively). *P. aeruginosa* isolates also demonstrated a reduction in swarming
118 motility as a result of co-incubation with amoeba, which varied over time in a non-linear
119 fashion ($F_{2, 534} = 7.597$, $p < 0.001$) (Fig 1.C). Swarming was significantly reduced in amoeba-
120 adapted isolates on days 3 and 24 ($F_{1, 534} = 21.73$, $p < 0.001$). Post hoc analysis showed that
121 after 3 days of co-incubation, the swarming distance of non-adapted isolates of *P. aeruginosa*
122 was twice that of amoeba-adapted isolates ($p > 0.001$). The swarming distance exhibited by
123 isolates derived from amoeba-adapted and non-adapted isolates on day 24 was further
124 reduced, with a significant reduction in swarming of the amoeba-adapted isolates compared
125 to non-adapted isolates ($p < 0.001$). On day 42 there was no significant difference between
126 the average swarming motility of isolates ($p = 0.189$).

127 Co-incubation of *P. aeruginosa* with *A. castellanii* had a significant effect on *P. aeruginosa*
128 biofilm formation ($F_{2, 354} = 15.7$, $p < 0.001$) (Fig. 1D). Post hoc analysis revealed no
129 differences between treatments for day 3 isolates ($p = 0.998$). However, amoeba-adapted
130 isolates from day 24 formed 10-fold less biofilm than the non-adapted isolates ($P < 0.001$).
131 Although the average biomass of biofilms formed by the amoeba-adapted isolates increased
132 after day 42, biofilm biomass remained 2-fold lower than that of the non-adapted isolates (F1,
133 354 = 29.6, $p < 0.001$).

134 *A. castellanii*-adapted *P. aeruginosa* isolates showed reduced production of pyoverdine
135 compared to non-adapted isolates ($F_{1, 174} = 45.74$, $P < 0.001$) (Fig. 1E). Although pyoverdine
136 production was reduced in both amoeba-adapted and non-adapted isolates ($F_{2, 174} = 12.08$, $p <$
137 0.001), the concentration of pyoverdine in supernatants from amoeba-adapted isolates from
138 day 3 isolates was reduced 2-fold compared to non-adapted isolates ($p < 0.001$) and was
139 further reduced on days 24, and 42 ($p < 0.001$). Rhamnolipid production of the amoeba-
140 adapted and non-adapted isolates varied on day 42 (Fig. 1F). Amoeba-adapted isolates

141 produced less rhamnolipid overall when compared with the non-adapted isolates ($t_{16} = 2.571$,
142 $p = 0.0205$)

143 **Amoeba-adapted *P. aeruginosa* isolates showed reduced virulence in *C. elegans***

144 Most of the phenotypes explored above play a role in the pathogenesis of *P. aeruginosa.*
145 Since the amoeba-adapted isolates showed marked reduction in the expression of multiple
146 virulence phenotypes, we tested for virulence in *C. elegans* fast and slow kill assays (Fig. 2A-
147 C). The day 3 amoeba-adapted isolates were significantly more toxic to nematodes compared
148 to non-adapted isolates, although nematodes exposed to isolates from both populations had a
149 median survival of 8 h ($p < 0.001$) (Fig. 2A). *C. elegans* feeding on day 42 isolates survived
150 longer than when feeding on isolates from the day 3. Furthermore, amoeba-adapted isolates
151 from day 42 were significantly less toxic to *C. elegans* compared to their non-adapted
152 counterparts in both fast kill (Fig. 2B) ($p < 0.001$) and slow kill (Fig. 2C) assays ($p < 0.001$).

153 **Genotypic changes in *A. castellanii*-adapted and non-adapted *P. aeruginosa***

154 To identify the underlying genetic basis for the attenuated virulence and reduced production
155 of virulence factors, three replicate (L1, L2, L3) populations of amoeba-adapted and non-
156 adapted *P. aeruginosa* from days 3 and 42 were sequenced. (Supplementary Data 1 and
157 Supplementary Data 1). In addition to whole population sequencing, 11 amoeba-adapted
158 single isolates and 3 non-adapted single isolates of *P. aeruginosa* from day 42 were
159 sequenced and analysed (Supplementary Data 3 and Supplementary Data 4). The number of
160 single nucleotide polymorphisms (SNPs), both synonymous (sSNP) and non-synonymous
161 (nsSNP) as well as insertions and deletions (INDELS) and intergenic mutations occurring in
162 amoeba-adapted and non-adapted populations and isolates were determined (Supplementary
163 Fig. 1A-B). More mutations were observed in both day 3 amoeba-adapted (N = 511) and non-
164 adapted (N = 601) populations than day 42 amoeba-adapted (N = 327) and non-adapted (N =

165 383) populations. This strongly suggests adaptive evolution of *P. aeruginosa* as random
166 mutations arise during early stages of adaptation and only beneficial mutations become fixed
167 in the later populations due to selection pressure. The days 3 and 42 adapted populations had
168 356 and 227 mutations in coding regions (sSNP, nsSNP and INDEL) corresponding to 270
169 and 181 genes, respectively. In contrast, days 3 and 42 non-adapted populations had 424 and
170 271 mutations in coding regions (sSNP, nsSNP and indel) corresponding to 282 and 198
171 genes, respectively. Days 3 and 42 adapted and non-adapted populations shared 22.9% (n =
172 84) and 28.3% (n = 106) mutated genes between them, respectively (Fisher exact test, p =
173 0.09) (Fig. 3A-B). There were more shared genes (n = 148, 36.6%) in day 3 adapted and non-
174 adapted populations compared to day 42 adapted and non-adapted populations (n = 73,
175 23.9%) (Fisher exact test, $p < 0.001$) (Fig. 3C-D).

176 Many mutations in known virulence-related genes were observed in both day 3 and day 42
177 adapted populations but not in the non-adapted populations (Table 1 and Fig. 4). A large
178 number of motility-related genes were mutated in adapted populations, most notably in
179 flagellar (*flgK, fleS, flgF, flgH*) and type IV pili genes (*pilI, pilM, pilN, pilR* and *pilT).* Genes
180 involved in pyoverdine synthesis (*pvdN*) as well as the quorum sensing-regulator, *lasR*, were
181 mutated only in day 42 adapted populations. Only 14 mutations that occurred in day 3
182 adapted lineages (L1, L2, L3) were maintained in the day 42 population (Supplementary Fig.
183 2). Among those persistent mutations, the mutational frequency increased from day 3 to day
184 42 in adapted populations for *flgK* (78.9% to 100% in L3 and 40% to 45.5% in L2), *fleS*
185 (11.3% to 84.4% in L2) and PA2069 (23% to 27.4% in L1). In contrast, 21 different
186 mutations were maintained from day 3 to 42 in non-adapted populations (Supplementary Fig.
187 3).

188 Mutational analysis of day 42 adapted (n=11) and non-adapted (n=3) single isolates of *P.*

189 *aeruginosa* revealed a total of 107 and 60 mutations affecting 51 and 25 genes respectively

190 (Supplementary Fig. 1B). Twenty four out of 51 genes (47%) mutated in day 42 adapted

191 isolates encoded virulence factors according to the virulence factor database

192 (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Pseudomonas). The mutated genes

193 encoding virulence factors were classified into different functions including motility, quorum

194 sensing, metabolism, and protease production (Table 2). Genes related to motility and

195 adherence, flagella (*flgF, flgH, flgK, fliS*) and type IV pili (*pilJ*, *pilM*, *pilN*, *pilT*), were mostly

196 mutated at 100% frequency. In addition, *lasR* was also mutated at 100% frequency. The

197 genes involved in motility and quorum sensing (*pilJ*, *pilM*, *pilT*, *flgK*, *fliS*, *fleS* and *lasR*)

198 harbor deletion mutations ranging from 1 to 399 bp.

199 **Adapted isolates showed increase competitive fitness and survival in amoeba and**

200 **macrophage**

201 To investigate whether adaptation with amoeba confers a fitness advantage to *P. aeruginosa*,

202 we mixed fluorescent-tagged amoeba-adapted and non-adapted isolates and grew them

203 together with amoeba. After 48 h of co-incubation, the proportion of amoeba-adapted cells

204 was always higher (amoeba-adapted::GFP ($F_{1,4} = 95.27$, $p = 0.000617$) and amoeba-

205 adapted::mCherry ($F_{1,4} = 11.85$, $p = 0.0262$) when competed with the reciprocally tagged

206 non-adapted strain, compared to no amoeba controls (Fig. 5A-B). In addition, the intracellular

207 survival of day 42 adapted and non-adapted isolates were determined using a modified

208 gentamicin protection assay. Intracellular CFUs 3 h after infection of non-adapted isolates in

209 amoeba were higher than amoeba-adapted CFUs, however, after 24 h the numbers of

210 surviving intracellular non-adapted cells had decreased and were comparable to the amoeba-

211 adapted numbers (Adaptation × Time $F_{1,32} = 14$, $p < 0.001$) (Fig. 5C).

212 **Adapted isolates exhibit reduced uptake by and enhanced survival within different**

213 **phagocytic cells**

214 In order to determine if the intracellular survival of amoeba-adapted isolates extends to other

215 phagocytic cell types, we compared the ability of adapted and non-adapted *P. aeruginosa* to

216 survive in the presence of macrophage and human neutrophils. When the assay was

217 conducted with macrophages, survival trends were similar to that observed with amoeba (Fig.

218 5D). There was a significant interaction of amoeba adaptation and incubation time

219 (Adaptation × Time $F_{4, 64} = 6.692$, $p < 0.001$), with a higher initial uptake of day 42 non-

220 adapted isolates compared to the amoeba-adapted isolates, resulting in higher initial

221 intracellular CFUs, followed by a constant decrease in viable intracellular numbers between 5

222 and 18 h post-infection. The day 42 amoeba-adapted isolates were initially taken up by

223 macrophage in lower numbers, and the number of viable intracellular CFUs did not decrease

224 to the same extent as the non-adapted isolates, resulting in comparable numbers at 18 h post-
225 infection. At 24 h post-infection, macrophage cells infected with non-adapted *P. aeruginosa*
226 exhibited morphological changes and appeared similar to those infected with the wild type
227 strain (Fig. 5E). Propidium iodide staining showed that many of these macrophages were
228 dead. In contrast, macrophage infected with amoeba-adapted *P. aeruginosa* exhibited a more
229 normal morphology, with fewer cells taking up the propidium iodide stain, suggesting
230 amoeba-adapted isolates were less toxic to the macrophage than the non-adapted isolates.
231 Assays with human neutrophils showed that, after 2 h infection, intracellular CFUs of non-
232 adapted isolates in neutrophils were higher than amoeba-adapted CFUs (Fig. 5F). Amoeba-
233 adapted isolates also showed increased survival in the presence of human neutrophils when
234 compared to non-adapted isolates (Fig. 5G).

235 **Chronic cystic-fibrosis isolates also exhibit enhanced survival and reduced uptake**

236 As late-stage amoeba-adapted isolates exhibit several phenotypes similar to chronic CF
237 isolates, we wanted to know whether CF isolates also behave similarly in terms of
238 intracellular survival and uptake by amoeba. We tested the uptake and intracellular survival
239 of five CF isolates (PA55, PA57, PA64, PA92 and PA100) in amoeba and compared with
240 PAO1. The results showed that the CF isolates showed reduced uptake and increased
241 intracellular survival similar to the amoeba-adapted isolates (Fig. 5H). All the CF isolates
242 tested showed reduced uptake by amoeba. The majority of the CF isolates (PA55, PA57 and
243 PA92) showed a significant increase in survival compared to PAO1.

244 Discussion

245 In this study, amoeba-adapted isolates displayed a reduction in many virulence phenotypes,
246 including loss of motility, reduction in pyoverdine and rhamnolipid production. These
247 changes in virulence phenotypes are very similar to phenotypes of *P. aeruginosa* isolates

248 from CF lineages, which include gains in mucoidy and antibiotic resistance, and loss of
249 secondary metabolites and motility (23). The parallel losses of motility and secondary
250 metabolites in amoeba-adapted isolates in this study are particularly striking and evoke the
251 question of whether the selective forces driving these traits are the same in both systems.

252 Strong negative selection against traits could occur due to host recognition and the need for
253 evasion by pathogens. In both cases, loss of motility appears to be a strong selection factor.
254 This is because both mammalian immune cells and amoeba can recognize and bind *P.*
255 *aeruginosa* flagella through surface receptors (26, 27). Furthermore, loss of motility has also
256 been shown to significantly reduce phagocytic uptake by mammalian immune cells (28).
257 Several findings presented here also support predator avoidance as a selection pressure.
258 Uptake and intracellular survival experiments with different phagocytic cells revealed that
259 non-adapted isolates exhibit chemotaxis and rapidly swim towards and are taken up by
260 amoeba and macrophage cells (Fig. 5C, D). However, we observed that amoeba-adapted
261 isolates do not attach to the surface of the amoeba. This is supported by experiments showing
262 reduced uptake by amoeba and macrophage (Fig 5C, D). Loss of flagella and motility is
263 therefore adaptive for the purpose of predator avoidance. Additionally, chemotaxis mutants in
264 PA3348 were detected in the population genomic data. The loss of chemotaxis in amoeba-
265 adapted strains would be consistent with predator avoidance.

266 Traits may also be lost if the cost of maintaining the traits outweigh the benefits. Loss of
267 pyoverdine in *P. aeruginosa* in chronic CF infections has been reported (29) and shown to be
268 driven by social selection (30). In these cases, non-pyoverdine producing cheats benefit by
269 retaining the pyoverdine receptor for uptake of pyoverdine and iron, without needing to
270 produce the metabolically expensive product. Only when extrinsic pyoverdine is completely
271 lost do mutations appear in receptor genes.

272 In our study, there were many *pvd* mutations, however, we did not observe any mutations in
273 the receptor genes. This suggests that social selection may also be occurring here. It is also
274 possible that other avenues of iron uptake are preferentially utilized (31), as iron uptake
275 occurs via *hemO* in late CF strains (32). Selection against pyoverdine production may be a
276 balance between the effect of the energy cost of pyoverdine synthesis, iron uptake and
277 oxidative stress as previously described. Thus, the cost of maintaining the traits may
278 outweigh the benefits, hence resulting in the selection against these genes.

279 In addition to the phenotypes described above, some of the most common mutations in
280 various models of infection and co-evolution studies occur in quorum sensing genes. For
281 example, mutations in *lasR* are common in CF isolates (33). Such mutations are also found in
282 co-evolution studies with *C. elegans*, where mutations in *lasR* and *rhlR* quorum sensing
283 genes were found to occur early in the adaptation process. The regulatory genes *lasR* and
284 *rhlR* control the expression of many virulence genes. In turn, *las* and *rhl* mutants have been
285 shown to be less virulent in models of wound infection (34). In contrast, no correlation
286 between *lasR* and loss of virulence was found in a later study investigating virulence using a
287 *Dictyostelium discoideum* host model and 13 CF isolates harboring mutations in *lasR* (35).
288 The study does not formally rule out an influence of *lasR* inactivation on virulence, although
289 they suggested that *lasR* mutations may represent only a minor factor in the evolution of
290 bacterial virulence. Mutations in *lasR* affect many downstream genes and quorum sensing
291 systems and it has been proposed that such pleiotropic adaptive mutations in global
292 regulatory genes are more likely to occur than multiple mutations in individual virulence
293 traits. This may account for the prevalence of *lasR* mutations in chronic CF mutations. In this
294 study, mutations in *lasR* occurred in only one of the three amoeba-adapted replicates,
295 suggesting that a stronger selection may be driving the loss of individual virulence traits in
296 the other amoeba-adapted populations.

297 Experiments with both amoeba and phagocytic immune cells in this study also suggest that
298 fitness gains from adapting to amoeba could be similarly conferred to interactions with
299 macrophages cells. In amoeba, macrophage and neutrophils, amoeba-adapted *P. aeruginosa*
300 isolates display reduced uptake and virulence. This demonstrates the overlaps in traits used
301 by *P. aeruginosa* to interact with these hosts. This work also highlights potential overlaps in
302 host-pathogens interaction processes, even between cells as diverged as mammalian
303 macrophage cells and single-celled amoeba. The *A. castellanii* genome contains homologues
304 of interferon-γ inducible lysosomal thiol reductase enzyme (GILT), interferon inducible
305 GTPase, and the NRAMP homologue, all of which play a role in antimicrobial defence in
306 mammalian cells (36). However, more research is needed to determine whether these
307 defences play a role in *P. aeruginosa* infection and whether *P. aeruginosa* possesses
308 mechanisms to evade such defences in order to survive intracellularly within both phagocytic
309 cell types.

310 Moreover, while CF macrophages are known to have impaired phagocytosis and bacterial
311 killing, this has been shown to be mainly due to the CF lung environment, inflammation,
312 expression of dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) in
313 macrophages and other defects associated with CFTR dysfunction in macrophages and other
314 immune cells (37, 38). In burn wound patients, T3SS effectors such as ExoS and ExoT were
315 also implicated in defective macrophage phagocytosis by disrupting host cell actin skeleton
316 (39). In these cases, the loss of flagella, pili and motility, as observed in our study, may not
317 be the primary driver of reduced uptake of *P. aeruginosa*. Together, this suggests that
318 reduced uptake of *P. aeruginosa* by predators or immune cells is important for long-term
319 survival and adaptation in amoeba and may provide survival advantages in macrophages.
320 However, the selection for this phenotype likely occurs through various mechanisms.

321 The loss of function in virulence genes in amoeba-adapted isolates likely leads to a
322 subsequent loss in virulence phenotypes. Similar decreases in acute virulence phenotypes also
323 occurs in *P. aeruginosa* strains isolated from chronically infected CF patients (23, 40, 41). *P.*
324 *aeruginosa* in experimental evolution experiments with *C. elegans* also evolved an attenuated
325 virulence phenotype after serial passages (14). This is in contrast to other pathogens where
326 virulence has been demonstrated to increase after serial passages (42), once again suggesting
327 that *P. aeruginosa* may adapt towards a more commensal and chronic lifestyle in co-
328 evolution processes.

329 In this study, it has been demonstrated that adaptation to a more commensal lifestyle may
330 also confer benefits in an infectious context for a generalist pathogen, as it is clear that
331 although amoeba-adapted cells are less virulent, they are still capable of invading and
332 colonizing *C. elegans*. Similarly, adapted CF strains have been shown to be equally as
333 capable as environmental strains of infecting a new host in a mouse model (43). Although
334 amoeba may be thought of as training grounds for the formation of virulence traits, they may
335 also be grounds for the selection for a more 'chronic' state of co-existence. The data
336 presented here supports the coincidental evolution hypothesis of virulence, where adaptation
337 to commensal habitats may coincidentally modulate virulence factors. Thus, it is adaptation
338 to environmental niches driving the evolution of virulence phenotypes and not interaction
339 with the human host.

340

341 ## Materials and methods

342 ### Organisms and growth conditions

343 *P. aeruginosa* strain DK1 used for this study was initially obtained from a Danish CF patient
344 (P30M0) (24). Unless otherwise stated, *P. aeruginosa* DK1 strain and population-derived

345 isolates were grown in 10 mL lysogeny broth (LB10, BD Biosciences, USA) at 37 °C with
346 shaking at 200 rpm. *A. castellanii* was obtained from the American Type Culture Collection
347 (ATCC 30234) and was routinely maintained axenically in peptone-yeast-glucose (PYG)
348 medium (20 g protease peptone, 5 g yeast extract, and 50 mL 2 M glucose L^{-1}) at room
349 temperature. Prior to use in experiments, *A. castellanii* was passaged and washed twice with
350 1 × phosphate buffered saline (PBS; Sigma-Aldrich, USA) solution to remove PYG media.
351 *C. elegans* N2 Bristol was maintained on nematode growth medium (NGM) (per liter; 2.5 g
352 Bacto-Peptone (BD Biosciences, USA), 3 g NaCl, 7.5 g agar, 1 mL 5 mg mL^{-1} cholesterol, 1
353 mL 1 M $MgSO_4$, 1 M $CaCl_2$, and 25 mL 1 M potassium phosphate buffer at pH 6) fed with *E.*
354 *coli* OP50. RAW 264.7 macrophage cell lines (ATCC TIB-71) were grown in Dulbecco's
355 modified Eagle Medium (DMEM; Thermo Fisher, USA) with 10 % fetal bovine serum (FBS)
356 at 37 °C with 5 % CO_2. Before use, cells were washed with PBS and treated with trypsin
357 briefly before gentle detachment by scraping. Cells were then centrifuged at 1000 × *g* for 1
358 min and resuspended in experimental media before use.

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

360 Overnight cultures of *P. aeruginosa* grown in LB10 medium was centrifuged at 4000 × *g* for
361 5 min and washed twice with 1 × M9 salts solution (Sigma-Aldrich, USA; per litre, 6.78 g
362 Na_2HPO_4, 3 g H_2PO_4, 1 g NH_4Cl, 0.5 g NaCl). *A. castellanii,* at a concentration of 1×10^3
363 cells mL^{-1}, was seeded onto the surface of 25 cm^2 tissue culture flasks with 0.2 µm vented
364 caps filled with 10 mL 1 × complete M9 salts + 0.01% glucose. To maintain a strong
365 selective pressure from amoeba, 100 µL of *A. castellanii* and *P. aeruginosa* was taken from
366 percussed, 3 day established flasks and added to new flasks containing *A. castellanii* every 3
367 day. Three biological replicates (L1, L2, L3) of *P. aeruginosa* with *A. castellanii* were
368 maintained and termed asamoeba-adapted populations and the derived isolates were termed
369 as amoeba-adapted isolates

370 In parallel with the co-incubation experiment, exact three biological replicates of *P.*
371 *aeruginosa* were also maintained without *A. castellanii* termed as non-adapted populations
372 and derived isolates were termed as non-adapted isolates. Briefly, *P. aeruginosa* was diluted
373 to a cell concentration of 1 $\times 10^2$ cells mL^{-1} and added to tissue culture flasks containing 10
374 mL 1 × complete M9 + 0.01 % glucose. From these flasks, 100 µL of 3-day-old established
375 *P. aeruginosa* culture was added to flasks containing fresh media every 3 day.

376 **Isolation of intracellular *P. aeruginosa***

377 On days 3, 24 and 42, flasks containing *A. castellanii* (amoeba-adapted) were percussed until
378 the amoebae detached and 1 mL of the culture media was filtered through a 3 µm cellulose
379 acetate membrane (Merck, Germany) to retain the *A. castellanii. A. castellanii* were
380 resuspended in 5 mL of 1 × M9 salts and pelleted at 4000 × g for 5 min before resuspension
381 in 100 µL 1 × M9 salts solution. *A. castellanii* were lysed by the addition of 100 µL of 1 %
382 Triton–X for 1 min, the mixture was then pelleted and washed twice with 900 µL of 1 × M9
383 salts. The cell pellet was resuspended in 1 mL of 70 % LB10 + 30 % glycerol and stored at -
384 80 °C. The same treatment was applied to the non-adapted *P. aeruginosa*.

385 **Phenotypic screening of amoeba-adapted and non-adapted isolates**

386 To facilitate phenotypic screening nine single isolates were randomly selected from each
387 amoeba-adapted and non-adapted populations on day 3, 24 and 42. Phenotypic changes were
388 determined by motility, biofilm, pyoverdine and rhamnolipid production assay as indicated
389 below.

390 **Biofilm assay**

391 To determine if adaptation with *A. castellanii* altered biofilm formation , the biomass of
392 attached cells was quantified by crystal violet staining as previously described (44). Briefly,
393 overnight cultures of each isolate were added to 96 well plates containing 100 µL 1 × M9 +

394 0.4 % glucose. Plates were incubated at room temperature with agitation at 80 rpm for 24 h.
395 To separate the suspended biomass from the attached biomass, each well was washed once
396 with 1 × PBS. Cells attached to the plate surface were stained with 200 µL of 0.3% crystal
397 violet and incubated for 20 min, after which unbound crystal violet was removed by washing
398 3 times with 1 × PBS. Crystal violet was liberated from the cells with 300 µL of 10 mL of
399 absolute ethanol. The absorbance of the crystal violet was measured with a
400 spectrophotometer at 590 nm (Infinite® M200, Tecan, Switzerland) in triplicate.

401 **Motility assays**

402 Twitching, swarming and swimming motility were assessed as previously described, using
403 motility agar (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 1 mM
404 $MgSO_4$,100 µM $CaCl_2$, 2 gL^{-1} Dextrose, 5 g L^{-1} casamino acids) containing 1, 0.5 or 0.3 %
405 wt vol^{-1} agarose (Bacto™ , BD Biosciences, USA), respectively (45, 46). Five milliliters of
406 motility agar were added into the wells of 6 well plates and dried under laminar flow for 1 h.
407 Isolates were inoculated into the center of the well using 10 µL pipette tips, either to the base
408 of the plate for assessment of twitching motility or mid-agar for assessment of swimming and
409 swarming. Twitching and swarming plates were incubated at room temperature for 48 h and
410 swimming plates were incubated for 24 h prior to imaging with a digital camera (Canon EOS
411 600D digital single-lens reflex (DSLR) mounted on a tripod, to allow for phenotypic
412 characterization of the resulting colonies and comparative endpoint twitch, swarm and swim
413 distances. Determination of the zone of motility was semi-quantitatively analyzed using
414 ImageJ image analysis software. Motility was assessed in triplicate (n = 3).

415 **Quantification of pyoverdine**

416 To determine if adaptation with amoeba (3, 24 and 42 d) affects the production of
417 pyoverdine, isolates were grown overnight in 1 mL LB10 medium. Cells were removed by

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

421 **Quantification of rhamnolipids**

422 The orcinol method (47) was used to quantify the production of rhamnolipid biosurfactant of
423 adapted and non-adapted isolates from the day 42 populations. Briefly, overnight *P.*
424 *aeruginosa* LB cultures were diluted to OD_{600} 0.01 in 25 mL of AB minimal media (48)
425 supplemented with 2 g glucose and 2 g casamino acids L^{-1} and grown overnight at 37 °C with
426 shaking at 200 rpm. The cell density was determined ($OD_{600\ nm}$) before filtration and
427 extraction of crude rhamnolipid from the supernatant two times using diethyl ether (7 mL).
428 The organic layer was collected, combined, and concentrated in a vacuum concentrator
429 (SpeedVac, Thermo Scientific) at 0 °C for 1 h followed by 2 h at 25 °C, until white solids
430 formed. The solids were resuspended in 500 µL of water and 50 µL of this solution was
431 mixed with 450 µL of freshly prepared orcinol (0.19 % in 53 % H_2SO_4). Samples were
432 incubated at 80 °C for 30 min and allowed to cool at room temperature for 15 min before
433 quantification of absorbance (OD_{421}). The absorbance was normalized to cell concentration
434 ($OD_{600\ nm}$) for each sample and a factor of 2.5 was applied to convert values from a rhamnose
435 standard curve to rhamnolipid concentration (49).

436 **Fluorescent tagging of isolates**

437 To prepare fluorescently-tagged amoeba-adapted and non-adapted *P. aeruginosa*, two
438 isolates were randomly selected from the day 42 populations and grown overnight at 37 °C in
439 LB broth. Electroporation was performed as previously described (50). One milliliter of *P.*
440 *aeruginosa* at a cell density of 1×10^8 cells mL^{-1} was pelleted and washed twice with 300
441 mM sucrose. The expression tag carrying plasmid pUC18-TR6K-mini-Tn7T-Gm-GFP (0.5

442 μg) (expresses a green fluorescent protein *gfp*; emission 488 nm/excitation 509 nm) (51) or
443 pUC18T-miniTn7T-Gm-Mcherry (0.5 μg) (expresses a red fluorescent protein mCherry
444 emission 587 nm/excitation 610 nm) (52) was mixed with 1 μg pTNS1 helper plasmid and
445 300 μL resuspended bacteria, in a 2-mm-gap electroporation cuvette. This mixture was
446 electroporated at 1.8kV/25μF/2100 Ω, 2.5kV cm^{-1} in a Gene Pulser apparatus (BIO-RAD,
447 Hercules, CA, USA). Cell recovery was performed in ice-cold super optimal broth with
448 catabolite repression media (SOC) (10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM
449 $MgSO_4$, 20 g L^{-1} tryptone, 5 g L^{-1} yeast extract + 2% glucose. Cells were incubated with
450 shaking for 3 h at 37 °C. One hundred microliters of culture were plated onto LB10 plates
451 supplemented with 200 ug mL^{-1} gentamicin to select for GFP and mCherry transformants.
452 Bacterial stocks were derived from a single transformed colony.

453 **Competition assays**

454 To determine if prior exposure of *P. aeruginosa* to amoeba increased competitiveness when
455 grown with amoeba, a competition assay was performed. with amoeba-adapted and non-
456 adapted isolates derived on day 42 and were fluorescently tagged as described above. *A.*
457 *castellanii*, at a cell concentration of 1×10^5 cells mL^{-1} was added to 24 well plates (Falcon)
458 in 450 μL 1 × M9 salts + 0.01 % glucose solution. Overnight cultures of GFP or mCherry-
459 labelled *P. aeruginosa* were grown in LB10 broth supplemented with 200 μg gentamicin at
460 37 °C with agitation at 200 rpm. The amoeba-adapted::gfp and non-adapted::mCherry isolate
461 or the amoeba-adapted ::mCherry and the non-adapted::gfp isolate were mixed in equal
462 proportion and added to the wells containing amoeba to a final bacterial cell concentration of
463 2×10^6 cells mL^{-1}. Each experiment was conducted in triplicate. The plates were incubated at
464 room temperature with agitation at 60 rpm for 48 h before imaging on a Zeiss Z1 inverted
465 wide field microscope. Acquired images were deconvoluted in Autoquant X3 (Bitplane)

466 before quantification of the relative red and green fluorescence in the field of view using
467 Imaris 8 (Bitplane).

468 **Uptake and intracellular survival of *P. aeruginosa* in macrophages**

469 To investigate the dynamics of uptake and intracellular survival of day 42 adapted and non-
470 adapted isolates within macrophages, overnight LB cultures of adapted and non-adapted *P.*
471 *aeruginosa* isolates were added to RAW264.7 macrophage cells (5×10^4 cells/well in 96 well
472 tissue culture plates) in DMEM without FBS at a multiplicity of infection (MOI) of 100:1.
473 The infected cells were incubated at 37°C with 5% CO2. After co-incubation for 1 h, the
474 media was removed and replaced with media containing 100 µg mL^{-1} gentamicin to kill
475 extracellular bacteria. Macrophage were washed with PBS and lysed at 3, 5, 7, 12 and 18 h
476 post-infection and CFU counts were performed to enumerate surviving intracellular cells.
477 Propidium iodide (ThermoFisher LIVE/DEAD Cell Viability kit) staining was done to
478 determine the state of the host cells 24 h post-infection.

479 **Uptake and intracellular survival of *P. aeruginosa* in the presence of neutrophils**

480 *P. aeruginosa* (4 adapted and 3 non-adapted) isolates from overnight culture were washed
481 once in PBS then diluted in PBS (OD=0.1, ~1×10^8) and resuspended in complete media
482 (RPMI + 2% heat inactivated autologous plasma) to experimental concentrations just prior to
483 infection. Neutrophils were isolated from whole blood, collected from healthy donors in
484 lithium heparin vacutainer tubes and separated using polymorphprep (axis shield) and
485 centrifugation. RBCs were hypotonically lysed and neutrophils washed in HBSS (without
486 Ca^+ or Mg^+). Neutrophils were counted and resuspended at their final concentration in
487 complete media. In a 96 well plate, neutrophils were added to wells for challenge
488 (neutrophil+) and complete medium added to control wells (neutrophil-). *P. aeruginosa* was
489 added to both PMN+ and PMN- wells at a MOI of 100:1 and incubated for 1 h at 37 °C, 5%

490 CO_2. After co-incubation for 1 h, bacterial survival was determined by serial dilution and
491 plating on LB for enumeration. Uptake was determined by media removal and replacement
492 with media containing 100 µg mL^{-1} gentamicin to kill extracellular bacteria. At the
493 experiment endpoint a sample of infection was taken and lysed in a new 96 well plate,
494 followed by serial dilution and plating onto LB agar. CFUs were determined by counting and
495 percent inoculum determined as (CFU of neutrophil + wells/CFU neutrophil - wells x 100).
496 Counts were performed in triplicate and results are the pooled Means ± SEM from individual
497 experiments using 3 different donors.

498 **Uptake and intracellular survival of *P. aeruginosa* in amoeba**

499 To investigate uptake and intracellular survival in amoeba, day 42 adapted and CF isolates
500 were used to infect *A. castellanii* at MOI of 100:1. Briefly, amoeba (10^5) were seeded onto 24
501 well plates in M9 medium and bacterial cells (10^7) were used to infect the amoeba. Following
502 co-incubation, the media was removed and replaced with media containing 100 µg mL^{-1}
503 gentamicin to kill extracellular bacteria. Amoeba were washed with M9 and lysed at 2 and 4h
504 post-infection and CFU counts were performed to enumerate surviving intracellular cells.
505

506 **Nematode survival assay**

507 To determine if *P. aeruginosa* adaptation to *A. castellanii* altered bacterial virulence, we
508 tested the survival of *C. elegans* sp. Bristol N2 after feeding on *P. aeruginosa*. Axenic *C.*
509 *elegans* were obtained via the egg-bleach synchronization method, plated onto NGM agar
510 and fed with heat killed *Escherichia coli* OP50. L4 stage worms were re-suspended in 1× M9
511 salts solution and 10 - 30 worms were drop plated onto 35 mm dishes containing 2 mL fast or
512 slow kill agar (53) containing lawns of pre-established amoeba-adapted or non-adapted
513 isolates. Plates were incubated at 22 °C and worm numbers were scored by microscopy at 0,
514 4, 8, 24 and 48 h for fast kill assays, and once per day for slow kill assays. Nematode toxicity

515 was tested using 9 randomly selected *P. aeruginosa* isolates from each treatment and from
516 times 3 and 42 d. Nematode survival assays were repeated twice independently, and each
517 experiment was performed in triplicate.

518 **Sequencing of *P. aeruginosa* populations and isolates and computational tools**

519 Amoeba-adapted and non-adapted *P. aeruginosa* populations and single isolates derived from
520 these populations were sequenced to determine genotypic changes that occurred in response
521 to co-adaptation with amoeba. Genomic DNA was extracted from the parental wild type
522 strain, and three replicates amoeba-adapted and non-adapted populations derived from day 3
523 and 42. In addition, a total of 11 adapted and 3 non-adapted isolates derived from adapted and
524 non-adapted populations from day 42 were also sequenced. Nine and three of this adapted
525 and non-adapted isolates repectively from day 42 were same those were also used in all the
526 phenotypic analysis. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen,
527 Venlo, Netherlands) according to manufacturer's instructions. Sequencing libraries were
528 prepared using the TruSeq DNA sample preparation kit (Illumina, San Diego, CA, USA), and
529 sequenced on a MiSeq (Illumina, USA). Reads were aligned to the *P. aeruginosa* PAO1
530 reference genome and genetic variants including nsSNP, sSNP, indel and intergenic
531 mutations were detected using breseq pipeline with polymorphism frequency cut off 0.1 (54).
532 The genetic variants were manually curated to filter out the mutations that were also present
533 in the parental strain.

534 **Competition and survival assays in phagocytic cells**

535 To determine the competitive fitness and intracellular survival in different phagocytic cells,
536 competition assay and intracellular survival assay were performed using amoeba,
537 macrophage and neutrophil. Details on the competition and intracellular survival assay can be
538 found in the Supplementary information.

Human ethics

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

Statistical analysis

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

Data availability

Sequence data related to this study are available under the bio-project accession number PRJNA753158.

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732 # Figure Legends

733 **Fig 1. Effect of co-incubation on virulence factor production** Twitching (A), swimming
734 (B) and swarming (C) motility, biofilm biomass (D) and pyoverdine production (E) of
735 amoeba-adapted (black) and non-adapted (grey) *P. aeruginosa* isolates derived from days 3,
736 24 and 42. Rhamnolipid production of day 42 adapted and non-adapted isolates (F). Data are
737 presented as means ± SEM. Statistical analyses for A, B, C, D and E were performed using
738 two-way ANOVA and Tukey's multiple comparisons test. For, F statistical analyses were
739 performed using unpaired t-test. Statistical significance is indicated by * $P<0.05$, ** $P<0.01$
740 and *** $P<0.001$.

741 **Fig. 2. Effect of co-incubation of *P. aeruginosa* with *A. castellanii* on virulence to *C.***
742 ***elegans*.** *C. elegans* survival curves after exposure to *P. aeruginosa* isolates derived from
743 amoeba-adapted (black) or non-adapted (grey) isolates taken from days 3 (A) or 42 (B) for 0,
744 4, 8, 24 and 48 h in a fast kill assay. (C) Percent survival of *C. elegans* exposed to day 42
745 amoeba-adapted and non-adapted isolates of *P. aeruginosa* over 7 days in a slow kill assay.
746 Statistical significance between adapted and non-adapted isolates was determined using log-
747 rank tests is indicated by *** $P<0.001$. Shaded area represents 95% CI.
748

749 **Fig 3. Unique and shared mutated genes in coding regions of different populations.** A
750 Venn diagram of the number and percentages of unique and shared genes in day 3 vs 42
751 adapted (A), and day 3 vs 42 non-adapted (B), day 3 adapted vs non-adapted (C) and day 42
752 adapted vs non-adapted (D) populations.

753 **Fig 4. Mutations in amoeba-adapted and non-adapted *P. aeruginosa* on days 3 and 42.**
754 The average mutational frequency of three replicates (L1, L2, L3) populations of day 3
755 adapted (A), day 42 adapted (B), day 3 non-adapted (C) and day 42 non-adapted (D)

756 populations are plotted along the *P. aeruginosa* genome. Different symbols represent
757 different types of mutation as depicted by the legend. The unique mutations (those detected in
758 either adapted or non-adapted populations) are blue while grey denotes mutations found in all
759 groups.

760 **Fig 5. Competition and intracellular survival assays of amoeba-adapted and non-**
761 **adapted isolates.** The fluorescence ratios of day 42 amoeba-adapted + A::GFP *P. aeruginosa*
762 mixed with non-adapted – A:: mCherry (A) and amoeba-adapted + A::mCherry with non-
763 adapted – A::GFP (B) after 48 h of incubation with (black bars) and without (grey bars) *A.*
764 *castellanii*. Intracellular survival of day 42 amoeba-adapted (black) and non-adapted (grey)
765 isolates as CFU ml^{-1} over time in a modified gentamicin protection assay (log-scale, n=3)
766 conducted with amoeba (C) and macrophage cells (D). Propidium iodide staining of raw
767 264.7 macrophage cells 24 h after infection with wild type, day 42 amoeba-adapted (A+) and
768 non-adapted (-A) *P. aeruginosa* (E). Images are shown with and without the fluorescence to
769 illustrate changes in cell morphology. Survival of day 42 amoeba-adapted (+ A) and non-
770 adapted (- A) strains following incubation with human neutrophils where bacterial uptake (F)
771 and survival (G) were determined. Intracellular number of CF isolates at 2 and 4 hours of
772 infection with amoeba were determined (H). The ratio of 4h/2h were used to determine the
773 significance of intracellular survival compared to PAO1 using Kruskal-wallis test. Data are
774 presented as means ± SEM. Groups were analysed by student t-test. Statistical significance is
775 indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$