

1 ***In vivo* microscopy reveals the impact of *Pseudomonas aeruginosa***
2 **social interactions on host colonization**

3 Chiara Rezzoagli^{1,2*}, Elisa T. Granato³, Rolf Kümmerli^{1,2*}

4 ¹Department of Plant and Microbial Biology, University of Zurich, Zurich,
5 Switzerland

6 ²Department of Quantitative Biomedicine, University of Zurich, Zurich,
7 Switzerland

8 ³Department of Zoology, University of Oxford, Oxford, United Kingdom

9

10 Running title: Bacterial social dynamics and host colonization

11

12 * Corresponding authors:

13 Chiara Rezzoagli or Rolf Kümmerli, Department of Quantitative Biomedicine,
14 University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.

15 Email: chiara.rezzoagli@uzh.ch (CR), rolf.kuemmerli@uzh.ch (RK).

16 Phone: +41 44 635 48 01.

17

18 Conflict of interest: The authors declare no conflict of interest.

19 **Abstract**

20 Pathogenic bacteria engage in social interactions to colonize hosts, which
 21 include quorum-sensing-mediated communication and the secretion of
 22 virulence factors that can be shared as “public goods” between individuals.
 23 While *in-vitro* studies demonstrated that cooperative individuals can be
 24 displaced by “cheating” mutants freeriding on social acts, we know less about
 25 social interactions in infections. Here, we developed a live imaging system to
 26 track virulence factor expression and social strain interactions in the human
 27 pathogen *Pseudomonas aeruginosa* colonizing the gut of *Caenorhabditis*
 28 *elegans*. We found that shareable siderophores and quorum-sensing systems
 29 are expressed during infections, affect host gut colonization, and benefit non-
 30 producers. However, non-producers were unable to cheat and outcompete
 31 producers. Our results indicate that the limited success of cheats is due to a
 32 combination of the down-regulation of virulence factors over the course of the
 33 infection, the fact that each virulence factor examined contributed to but was
 34 not essential for host colonization, and the potential for negative-frequency
 35 dependent selection. Our findings shed new light on bacterial social
 36 interactions in infections and reveal potential limits of therapeutic approaches
 37 that aim to capitalize on social dynamics between strains for infection control.

38 Introduction

39 During infections, pathogenic bacteria secrete a wide range of extracellular
40 virulence factors to colonize and grow inside the host [1, 2]. Secreted
41 molecules include siderophores for iron scavenging, signaling molecules for
42 quorum sensing (QS), toxins to attack host cells, and matrix compounds for
43 biofilm formation [3–6]. *In-vitro* studies have shown that extracellular virulence
44 factors can be shared as “public goods” between cells, and thereby benefit
45 individuals other than the producing cell [7–9]. There has been enormous
46 interest in understanding how this form of bacterial cooperation can be
47 evolutionarily stable since secreted public goods can be exploited by non-
48 cooperative mutants called “cheats”, which do not engage in cooperation yet
49 still benefit from the molecules produced by others [10–12].

50

51 There is increasing evidence that social interactions and cooperator-cheat
52 dynamics might also matter within hosts [9, 13, 14]. For instance, in controlled
53 infection experiments engineered non-producers, deficient for the production
54 of specific virulence factors, could outcompete producers and thereby reduce
55 virulence [15–19], but in other cases the success of non-producers was
56 compromised [20, 21]. Other studies have followed chronic human infections
57 within patients over time and reported that virulence-factor-negative mutants
58 emerge and spread, with the mutational patterns suggesting cooperator-cheat
59 dynamics [7, 22, 23]. These findings spurred ideas of how social interactions
60 within hosts could be manipulated for therapeutic purposes [14, 24, 25].
61 Suggested approaches include: inducing cooperator-cheat dynamics to steer
62 infections towards lower virulence [7, 26]; introducing less virulent strains with

63 medically beneficial alleles into established infections [24]; and targeting
64 secreted virulence factors to control infections and constrain the evolution of
65 resistance [25, 27–31].

66

67 However, all these approaches explicitly rely on the assumption that the social
68 traits of interest are: (i) expressed inside hosts; (ii) important for host
69 colonization; (iii) exploitable; and (iv) induce cooperator-cheat dynamics as
70 observed *in vitro* [9] – assumptions that have not yet been tested in real time
71 inside living hosts. Here, we explicitly test the importance of bacterial social
72 interactions within hosts by using *in-vivo* fluorescence microscopy to monitor
73 bacterial virulence factor production, host colonization and strain interactions,
74 using the opportunistic pathogen *Pseudomonas aeruginosa* and its nematode
75 model host *Caenorhabditis elegans* [32–34].

76

77 *C. elegans* naturally preys on bacteria [35]. While most bacteria are killed
78 during ingestion, a small fraction of cells survives [36], which can, in the case
79 of pathogenic bacteria, establish an infection in the gut [37]. *P. aeruginosa*
80 deploys an arsenal of virulence factors that facilitate successful host
81 colonization [38]. For example, the two siderophores pyoverdine and pyochelin
82 scavenge host-bound iron during acute infections to enable pathogen growth
83 [6, 39–41]. *P. aeruginosa* further secretes the protease elastase, the toxin
84 pyocyanin, and rhamnolipid biosurfactants to attack host tissue [7, 42, 43].
85 Production of these latter virulence factors only occurs at high cell densities
86 and is controlled by the Las and the Rhl QS-systems [44]. Because both QS-

87 regulated virulence factors and siderophores were shown to be involved in *C.*
88 *elegans* killing [37, 45–48], we used them as focal traits for our study.

89

90 To tackle our questions, we first conducted experiments with fluorescently
91 tagged *P. aeruginosa* bacteria (PAO1) to follow infection dynamics, from the
92 first uptake through feeding up to a progressed state of gut infection. We then
93 constructed promoter-reporter fusions for genes involved in the synthesis of
94 the two siderophores (pyoverdine and pyochelin) and the two QS-regulators
95 (LasR and RhIR) to track *in vivo* virulence factor gene expression during host
96 colonization. Subsequently, we used mutant strains deficient for virulence
97 factors to determine whether they show compromised colonization abilities.
98 Finally, we followed mixed infections of wildtype and mutants over time to
99 determine the extent of strain co-localization in the gut, and to test whether
100 secreted virulence factors are indeed exploitable by non-producers in the host.

101

102 **Material and methods**

103 **Strain and bacterial growth conditions**

104 Bacterial strains, primers and plasmids used in this study are listed in the
105 Supplementary Tables S1-S3. Details on strain construction are given in the
106 Supplementary Methods. For all experiments, overnight cultures were grown
107 in 8 ml Lysogeny broth (LB) in 50 ml tubes, incubated at 37°C, 220 rpm for 18
108 hours, washed with 0.8% NaCl solution and adjusted them to OD₆₀₀ = 1.
109 Nematode Growth Media (NGM) plates [0.25% Peptone, 50 mM NaCl, 25mM
110 [PO₄⁻], 5 µg/ml Cholesterol, 1mM CaCl₂, 1mM MgSO₄ supplemented with
111 1.5% agar, 6 cm diameter] were seeded with 50 µl of bacterial culture and

112 incubated at 25°C for 24 hours. All *P. aeruginosa* strains used in this study
113 showed equal growth on NGM exposure plates (Supplementary Figure S1).
114 Peptone was purchased from BD Biosciences, Switzerland, all other
115 chemicals from Sigma Aldrich, Switzerland.

116

117 **Nematode culture**

118 We used the temperature-sensitive, reproductively sterile *C. elegans* strain
119 JK509 (*glp-1(q231)* III). This strain reproduces at 16°C, but does not develop
120 gonads and is therefore sterile at 25°C. Worms were maintained fertile at 16°C
121 on High Growth Media (HGM) agar plates (2% Peptone, 50 mM NaCl, 25mM
122 [PO₄], 20 µg/ml Cholesterol, 1mM CaCl₂, 1mM MgSO₄) seeded with the
123 standard food source *E. coli* strain OP50 [49]. For age synchronization, plates
124 were washed with sterile distilled water and adult worms were killed with
125 hypochlorite-sodium hydroxide solution to isolate eggs [49]. Eggs were placed
126 in M9 buffer (20 mM KH₂PO₄, 40 mM Na₂HPO₄, 80 mM NaCl, 1 mM MgSO₄)
127 and incubated at 16°C for 16-18 hours to hatch. Then, L1 larvae were
128 transferred to OP50-seeded HGM plates and incubated at 25°C for 28 hours
129 to reach L4 developmental stage. Worms and OP50 bacteria were provided by
130 the Caenorhabditis Genetic Center (CGC), which is supported by the National
131 Institutes of Health - Office of Research Infrastructure Programs (P40
132 OD010440).

133

134 ***C. elegans* infection protocol**

135 Synchronized L4 worms were washed from HGM plates with M9 buffer + 50
136 µg/ml kanamycin (M9-Kan), and washed three times with M9-Kan for worm

137 surface-disinfection. Viable worms were further separated from any debris by
138 sucrose flotation [50] and rinsed three time in M9 buffer to remove sucrose.
139 The worm handling protocol for the main experiments is depicted in Figure 1A.
140 Specifically, approximately 200 worms were moved to seeded NGM plates
141 and incubated for 24 hours at 25°C. After this period of exposure to
142 pathogens, infected worms were extensively washed with M9 buffer + 50
143 µg/ml chloramphenicol (M9-Cm) followed by M9 buffer, subsequently
144 transferred to individual wells of a 6-well plate filled with sterile M9 buffer + 5
145 µg/ml cholesterol (M9+Ch Buffer) where they were kept up to 48 hours post
146 exposure (hpe) and imaged after 0, 6 and 30 hpe.

147

148 **Nematode survival assay**

149 Our goal was to observe infections inside living hosts. To verify that worms
150 stayed alive during the experiment (up to 48 hpe), we tracked the survival of
151 infected population (50-90 worms) in M9-Ch buffer. Worms were observed for
152 motility at 0, 24 and 48 hpe, by prodding them with a platinum wire. Worms
153 were considered dead when they no longer responded to touches. Each
154 bacterial strain was tested in three replicates and three independent
155 experiments were carried out. We used *E. coli* OP50 as a negative control for
156 killing. During this observation period, worms experienced only negligible
157 killing by the colonizing bacteria, and we found no significant difference in
158 killing between the non-pathogenic *E. coli* food strain and the *P. aeruginosa*
159 strains (Supplementary Figure S2).

160

161 **Microscopy setup and imaging**

For microscopy, we picked individual worms from the M9+Ch buffer and paralyzed them with 25 mM sodium azide before transferring them to an 18-well μ -slide (Ibidi, Germany). All experiments were carried out at the Center for Microscope and Image Analysis of the University Zürich (ZMB). For the colonization experiment, images were acquired on a Leica LX inverted widefield light microscope system with Leica-TX2 filter cube for mCherry (emission: 560 ± 40 nm, excitation: 645 ± 75 nm, DM = 595) and Leica-DFC-350-FX, cooled fluorescence monochrome camera (resolution: 1392×1040 pixels) for image recording (16-bit color depth). For gene expression experiments, microscopy was performed on the InCell Analyzer 2500HS (GE Healthcare) automated imaging system, using polychroic beam splitter BGRFR_2 (for mCherry, excitation: 575 ± 25 nm, emission: 607.5 ± 19 nm) and PCO – sCMOS camera (resolution: 2048×2048 pixels, 16-bit).

175

176 **Image processing and analysis**

To extract fluorescence measurements from individual worms, images were segmented into objects and background, using an automated image segmentation workflow with *ilastik* software [51]. Segmented images were then imported in *Fiji* [52] to determine the fluorescence intensity (as “Raw Integrated Density”, i.e. the sum of pixels values in the selection) and area of each worm. Images obtained from the InCell microscope entailed 64 frames (8×8 grid) with 10% overlap. These frames were stitched together using a macro-automated version of the Stitching plugin in *Fiji* [53] prior to segmentation and analysis. To correct for background and host-tissue autofluorescence, we imaged, at each time point, worms infected with non-

187 fluorescent strains (i.e. OP50 or PAO1), and used the mean intensity of these
188 control infections to subtract background fluorescence values from worms
189 infected with fluorescent strains.

190

191 Competition assay in the host

192 For *in-vivo* competitions between PAO1-*mCherry* and PAO1 Δ *pvdD* Δ *pchEF* or
193 PAO1 Δ *asR*, overnight monocultures were washed twice with 0.8% NaCl
194 solution, adjusted to OD₆₀₀ = 1 and mixed at a 1:1 ratio. To control for fitness
195 effects of the mCherry marker, we also competed PAO1-*mCherry* against the
196 untagged PAO1. NGM plates were then seeded with 50 μ l of mixed culture
197 and incubated at 25°C for 24 hours. Worms were exposed to the mix for 24
198 hours and then recovered as previously described. After 6 and 48 hours post-
199 exposure, individual worms were picked, immobilized with sodium azide and
200 washed for 5 minutes with M9 + 0.003% NaOCl. Worms were washed twice
201 with M9 buffer. We then transferred each individual worm to a 1.5 ml screw-
202 cap micro tube (Sarstedt, Switzerland) containing sterilized glass beads (1 mm
203 diameter, Sigma Aldrich). Worms were disrupted using a bead-beater
204 (TissueLyser II, QIAGEN, Germany), shaking at 30 Hz for 1.5 min before
205 flipping the tubes and shaking for an additional 1.5 min to ensure even
206 disruption (adapted from [54]). Tubes were then centrifuged at 2000 x g for 2
207 min, the content was re-suspended in 200 μ l of 0.8% NaCl and plated on two
208 LB 1.2 % agar plates for each sample. Plates were incubated overnight at
209 37°C and left at room temperature for another 24 h to allow the fluorescent
210 marker to fully mature. We then distinguished between fluorescent and non-
211 fluorescent colonies using a custom built fluorescence imaging device (*Infinity*

212 3 camera, Lumenera, Canada). We then calculated the relative fitness of
213 PAO1-*mCherry* as $\ln(v) = \ln\{[a_{48} \times (1 - a_6)] / [a_6 \times (1 - a_{48})]\}$, where a_6 and a_{48} are the
214 frequency of PAO1-*mCherry* at 6 and 48 hours after recovery, respectively
215 [55]. Values of $\ln(v) < 0$ or $\ln(v) > 0$ indicate whether the frequency of PAO1-
216 *mCherry* increased ($\ln(v) < 0$) or decreased ($\ln(v) > 0$) relative to its competitor.

217

218 **Co-localization analysis**

219 To determine the degree of co-localization of two different bacterial strains in
220 the host, we transferred nematodes to NGM plates seeded with a 1:1 mix of
221 PAO1-*gfp* with either PAO1-*mCherry*, PAO1 Δ *pvdD* Δ *pchEF*-*mCherry*, or
222 PAO1 Δ *lasR*-*mCherry*. After a 24 hours grazing period, we picked single
223 worms and imaged both mCherry- and GFP channels, using the InCell
224 Analyzer 2500HS microscope. We used *Fiji* to straighten each worm with the
225 *Straighten* plugin [56], and extracted fluorescence intensity values in the GFP
226 and mCherry channels for each pixel from tail ($X = 0$) to head ($X = 1$) of the
227 worm. To ensure that we only measure areas where bacteria were present, we
228 restricted our analysis to the region of the worm gut, where bacterial
229 colonization takes place. We then calculated Spearman correlation coefficients
230 between the fluorescent signals, as a proxy for strain co-localization using
231 RStudio v. 3.3.0 [57].

232

233 **Statistical analysis**

234 All statistical analyses were performed with RStudio. We used Pearson
235 correlations to test for associations between PAO1-*mCherry* fluorescence
236 intensities and (a) recovered bacteria from the gut; and (b) total bacterial load

in mixed infections. We used analysis of variance (ANOVA) to compare fluorescence values between observation times, strains and for comparisons to non-fluorescent controls. *P*-values were corrected for multiple comparisons using the post-hoc Tukey HSD test. To compare promoter expression data between PAO1 WT and mutant strains, and to compare relative fitness values between competitors in the competition assay, we used Welch's two-sample *t*-test. To measure co-localization, we calculated the Spearman correlation coefficient ρ between the intensity of mCherry and GFP signals across the worm gut, and used ANOVA to test for differences between treatments.

Results

PAO1 colonization dynamics in the *C. elegans* gut

For all infection experiments, we followed the protocol depicted in Figure 1A-C. We first exposed worms to *P. aeruginosa* for 24 hours on NGM plates. Subsequently, worms were removed, washed, and treated with antibiotics to kill external bacteria. We then imaged infected worms under the microscope at different time points and quantified bacterial density and gene expression using fluorescent mCherry markers. We first confirmed that mCherry fluorescence is a suitable proxy for the number of live bacteria in *C. elegans*, by comparing fluorescence intensities in whole worms (Figure 1B) to the number of live bacteria recovered from the worms' gut. Fluorescence intensity values positively correlated with the bacterial load inside the nematodes, both immediately after recovering the worms from the exposure plates and at 6 hours post exposure (hpe; Supplementary Figure S3, Pearson correlation coefficient at 0 hpe: $r = 0.49$, $t_{28} = 3.02$, $p = 0.0053$; at 6 hpe: $r = 0.713$, $t_{23} =$

262 4.88, $p < 0.0001$). As our goal was to image infections in living hosts, we
263 further confirmed that worms stayed alive during the observation period
264 (Supplementary Figure S2).

265

266 When following host colonization by PAO1-*mCherry* over time, we observed
267 that immediately after removal from the exposure plate, worms carried large
268 amounts of bacteria in their gut (Figure 1D). Subsequently, bacterial load
269 significantly declined when worms were kept in buffer for 6 hours (ANOVA: t_{391}
270 = -8.55, $p < 0.001$) and then remained constant for the next 24 hours (t_{391} =
271 0.61, $p = 0.529$). This pattern suggests that a large number of bacteria are
272 taken up during the feeding phase, followed by the shedding of a high
273 proportion of cells, leaving behind a fraction of live bacteria that establishes an
274 infection and colonizes the worm gut.

275

276 **PAO1 expresses siderophore biosynthesis genes and QS regulators in** 277 **the host**

278 We then examined whether genes involved in the synthesis of pyoverdine
279 (*pvdA*) and pyochelin (*pchEF*), and the genes encoding the QS-regulators
280 *lasR* and *rhIR*, are expressed inside hosts. Worms were exposed to four
281 different PAO1 strains, each carrying a specific promoter-*mCherry* fusion.
282 Imaging after the initial uptake phase (0 hpe) revealed that, with the exception
283 of *pchEF*, all genes were significantly expressed in the host (Figure 2;
284 ANOVA, comparisons to the non-fluorescent control, for *pvdA*: $t_{754} = 4.23$, $p <$
285 0.001 ; for *pchEF*: $t_{754} = 0.74$, $p = 0.461$; for *lasR*: $t_{754} = 2.96$, $p = 0.003$; for
286 *rhIR*: $t_{754} = 10.37$, $p < 0.001$). Although fluorescence intensity declined over

time (linear model, $F_{1,1795} = 48.98$, $p < 0.001$), we observed that apart from *pchEF*, all genes were still significantly expressed during the subsequent colonisation of the host at 30 hpe. (Figure 2; ANOVA, for *pvdA*: $t_{754} = 4.87$, $p < 0.001$; for *pchEF*: $t_{754} = 0.684$, $p = 0.461$; for *lasR*: $t_{754} = 3.01$, $p = 0.003$; for *rhIR*: $t_{754} = 16.68$, $p < 0.001$).

Regulatory links between social traits operate inside the host

We know that regulatory links exist between the virulence traits studied here. While pyoverdine synthesis suppresses pyochelin production under stringent iron limitation [58], the Las-QS system positively activates the Rhl-QS system [44]. To test whether these links operate inside the host, we measured gene expression of each trait in the negative background of the co-regulated trait (Figure 3). For *pvdA*, we observed significant gene expression levels in both the wildtype PAO1 and the pyochelin-deficient PAO1 Δ *pchEF* strain (Figure 3A), albeit the overall expression was slightly reduced in PAO1 Δ *pchEF* (t-test, $t_{253} = 8.67$, $p < 0.001$). For *pchEF*, expression patterns confirm the suppressive nature of pyoverdine: the pyochelin synthesis gene was not expressed in the wildtype but significantly upregulated in the pyoverdine-deficient PAO1 Δ *pvdD* strain (Figure 3B; $t_{296} = -19.68$, $p < 0.001$). For *lasR*, we found that gene expression was not significantly different in wildtype PAO1 compared to the Rhl-negative mutant PAO1 Δ *rhIR*, confirming that the Las-QS system is at the top of the hierarchy and not influenced by the Rhl-system (Figure 3C; $t_{211} = -1.50$, $p = 0.136$). Conversely, the expression of *rhIR* was strongly dependent on a functional Las-system, and therefore only expressed in PAO1, but repressed in the Las-negative mutant PAO1 Δ *lasR* (Figure 3D;

312 $t_{156} = 19.04$, $p < 0.001$). These results show that (i) iron-limitation is strong in
313 *C. elegans* as PAO1 primarily invests in the more potent siderophore
314 pyoverdine; (ii) pyochelin can have compensatory effects when pyoverdine is
315 lacking; and (iii) the loss of the Las-system leads to the concomitant collapse
316 of the Rhl-system.

317

318 **Virulence-factor-negative mutants show trait-specific deficiencies in host** 319 **colonization**

320 To examine whether the ability to produce shared virulence factors is
321 important for initial bacterial uptake and host colonization, we exposed
322 *C. elegans* to five isogenic mutants of PAO1-*mCherry*, either impaired in the
323 production of pyoverdine ($\Delta pvdD$), pyochelin ($\Delta pchEF$), both siderophores
324 ($\Delta pvdD\Delta pchEF$), the QS receptor LasR ($\Delta lasR$), or the QS receptor RhlR
325 ($\Delta rhIR$). After the feeding phase, the bacterial load of the wildtype and all three
326 siderophores mutants were equally abundant inside hosts, whereas bacterial
327 load was significantly reduced for the two QS-mutants compared to the
328 wildtype (Figure 4A; ANOVA, significant variation among strains $F_{5,736} = 10.50$,
329 $p < 0.001$; post-hoc Tukey test for multiple comparisons: $p > 0.05$ for all
330 siderophore mutants, $p = 0.021$ for PAO1 $\Delta lasR$, $p < 0.001$ for PAO1 $\Delta rhIR$).

331

332 As previously described for PAO1 colonization (Figure 1D), we observed that
333 the bacterial load of all strains declined at 6 hpe (Supplementary Figure S4)
334 and 30 hpe (Figure 4B) following worm removal from the exposure plates. This
335 decline was significantly more pronounced for the double-siderophore
336 knockout PAO1 $\Delta pvdD\Delta pchEF$ than for the wildtype (Figure 4B; ANOVA, post-

337 hoc Tukey test $p < 0.001$). In contrast, mutants deficient in pyochelin
 338 (PAO1 $\Delta pchEF$) and RhIR (PAO1 $\Delta rhIR$) production showed a significantly
 339 higher ability to remain in the host than the wildtype (Figure 4B; ANOVA, post-
 340 hoc Tukey test $p < 0.001$ for both strains). Taken together, our findings suggest
 341 that the two siderophores can complement each other, and that only the
 342 siderophore double mutant and the LasR-deficient strain have an overall
 343 disadvantage in colonizing worms.

344

345 **Mixed communities are formed inside hosts, but exploitation of social**
 346 **traits is constrained**

347 Given our findings on colonization deficiencies, we reasoned that the
 348 siderophore-double mutant (PAO1 $\Delta pvdD\Delta pchEF$) and the Las-deficient
 349 mutant (PAO1 $\Delta lasR$) could act as cheats and benefit from the exploitation of
 350 virulence factors produced by the wildtype in mixed infections. To test this
 351 hypothesis, we first competed the PAO1-*mCherry* strain against the untagged
 352 wildtype in the host, and found that the mCherry tag had a small negative
 353 effect on PAO1 fitness (Figure 5A; one sample t-test, $t_{24} = -4.12$, $p < 0.001$).
 354 We then competed PAO1-*mCherry* against the two putative cheats and found
 355 that neither of them could gain a significant fitness advantage over the
 356 wildtype, but also did not lose out (Figure 5A; ANOVA, $F_{2,70} = 0.517$, $p =$
 357 0.598). These results indicate that virulence-factor-negative mutants, initially
 358 compromised in host colonization, can indeed benefit from the presence of the
 359 wildtype producer, but not to an extent that would allow them to increase in
 360 frequency and displace producers.

361

362 Since our mono-infection experiments showed that the wildtype can maintain
 363 higher bacterial loads in the worms compared to the two mutants (Figure 4B),
 364 we hypothesized that worms, which have initially taken up higher frequencies
 365 of the wildtype relative to the mutant should carry increased bacterial loads in
 366 the gut. We found this prediction to hold true at 6 hpe in mixed infections with
 367 the two non-producers, but not in the control mixed infections with the
 368 untagged wildtype (Figure 5B, 6hpe; Pearson correlation coefficient, for mixed
 369 infection with PAO1 Δ lasR: $r = 0.54$, $t_{17} = 2.67$, $p = 0.016$; with
 370 PAO1 Δ pvdD Δ pchEF: $r = 0.40$, $t_{17} = 1.77$, $p = 0.031$; with control PAO1: $r =$
 371 0.12 , $t_{17} = 0.47$, $p = 0.639$). These correlations disappeared at the later
 372 colonization stage (Figure 5B, 48 hpe; Pearson correlation coefficient $r < 0$, p
 373 > 0.05 for all strains). The loss of these correlations indicates that rare
 374 producers experienced a selective advantage during competition and
 375 increased in relative frequency, while common producers might have lost and
 376 decreased in frequency.

377

378 **Strain co-localization is generally high within the host, but varies** 379 **substantially across individuals**

380 *In-vitro* studies have shown that spatial proximity of cells is crucial for efficient
 381 compound sharing [59, 60]. We thus assessed the co-localization of strains in
 382 mixed infections inside the gut (Figure 6). We found that all worms were
 383 colonized by both strains, and that the level of co-localization ρ (from tail to
 384 head) was generally high, although it varied substantially across individuals
 385 (Figure 6A-B). Similar co-localization patterns emerged for all three strain
 386 combinations tested, highlighting that the type of competitor did not influence

the degree of strain co-localization in the host gut (Figure 6C; ANOVA, $F_{2,102} = 2.17$, $p = 0.119$). While our measure of co-localization has some limitations as it does not reveal physical proximity at the single cell level, and is based on a 2D projection of a 3D organ, it clearly suggests that competing cells are close to one another, and that social interactions could occur between them.

Discussion

We developed a live imaging system that allows us to track host colonization by pathogenic bacteria (*P. aeruginosa*) and their expression of virulence factors inside hosts (*C. elegans*). We used this system to focus on the role of secreted virulence factors, which can be shared as public goods between bacterial cells, and examined competitive dynamics between virulence factor producing and non-producing strains in the host. We found that siderophores (pyoverdine and pyochelin) and the Las and Rhl QS-systems (i) are expressed inside the host; (ii) affect the ability to colonize and reside within the nematodes; (iii) allow non-producers to benefit from virulence factors secreted by producers in mixed infections; but (iv) do not allow non-producers to cheat and outcompete producers. Our results have implications for both the understanding of bacterial social interactions within hosts, and therapeutic approaches that aim to manipulate social dynamics between strains for infection control.

Numerous *in-vitro* studies have shown that bacterial cooperation can be exploited by cheating mutants that no longer express the social trait, but benefit from the cooperative acts performed by others [3, 11, 12, 55, 61–65].

412 These findings contrast with our observations that the spread of non-
 413 producers was constrained within infections. There are multiple ways to
 414 explain this constraint. First, increased spatial structure can limit the diffusion
 415 of secreted metabolites and lead to the physical separation of cooperators and
 416 cheats [63]. Both effects result in metabolites being shared more locally
 417 among cooperative individuals. The physical separation of strains seemed to
 418 explain the results of Zhou *et al* [20], where QS-mutants of *Bacillus*
 419 *thuringiensis* infecting caterpillars could not exploit metabolites from
 420 producers. Conversely, physical separation seemed low in our study system
 421 (Figure 6), and therefore unlikely explains why cheats could not spread. While
 422 we solely focussed on proximity patterns inside hosts, it is important to note
 423 that processes at the meta-population level such as bottlenecking [54, 66], can
 424 reduce the probability of different strains ending up in the same host, and thus
 425 further compromise cheat success.

426

427 Second, negative frequency-dependent selection could explain why the
 428 spread of virulence factor negative mutants is constrained [55]. This scenario
 429 predicts that cheats only experience a selective advantage when rare, but not
 430 when common. The reasoning is that non-producers can only efficiently exploit
 431 public goods when surrounded by many producers. Our competition
 432 experiments indeed provide indirect evidence for negative frequency-
 433 dependent selection in the nematode gut (Figure 5B). Specifically, we
 434 observed that bacterial load was reduced when producers occurred at low
 435 frequency early during infection (6 hpe), a result confirming that non-producers
 436 are worse host colonizers than producers. These correlations disappeared

437 during the competition period (48 hpe), indicating that rare producers might
438 have experienced a selective advantage and increased in relative frequency,
439 while common producers lost and decreased in frequency.

440

441 Third, the relatively low bacterial density in the gut could further compromise
442 the ability of non-producers to cheat (Figure 1D, 5B). Low cell density restricts
443 the sharing and exploitation of secreted compounds [67, 68]. Mechanisms
444 responsible for the low bacterial density in the gut (Figure 1D, 5B) could
445 include the peristaltic activity of the gut, expelling a part of the pathogen
446 population and the host immune system, killing a fraction of the bacteria [69].

447

448 Fourth, our analysis reveals that, although siderophores and QS-systems play
449 a role in host colonization, they are not essential (Figure 4). Moreover, the
450 expression of pyoverdine and QS-systems declined over time (Figure 2).
451 These two observations indicate that the benefits of cheating might be fairly
452 low, and that the costs of virulence factor production are reduced at later
453 stages of the infection. Thus, bacteria might switch from production to
454 recycling of already secreted public goods [70, 71], an effect that can hamper
455 the spread of cheats.

456

457 Finally, we show that the regulatory linkage between traits is an important
458 factor to consider when predicting the putative advantage of non-producers
459 [72, 73]. For instance, *P. aeruginosa* pyoverdine-negative-mutants
460 upregulated pyochelin production to compensate for the lack of their primary
461 siderophore (Figure 3). Thus, if pyoverdine-negative mutants evolve *de novo*,

462 their spread as cheats could be hampered because they invest in pyochelin as
 463 an alternative siderophore [74]. For QS, meanwhile, we observed that the
 464 absence of a functional Las-system resulted in the concomitant collapse of the
 465 Rhl-system. Although *lasR* mutants could be potent cheats, as they are
 466 deficient for multiple social traits, their spread might be hampered because
 467 QS-systems also regulate non-social traits, important for individual fitness [75].

468

469 When relating our insights to previous studies, it turns out that earlier work
 470 produced mixed results with regard to the question whether siderophore- and
 471 QS-deficient mutants can spread within infections. While Harrison *et al.* ([15,
 472 21]; pyoverdine, *P. aeruginosa* in *Galleria mellonella* and *ex-vivo* infection
 473 models) and Zhou *et al.* ([20]; QS, *B. thuringiensis* in *Plutella xylostella*)
 474 showed that the spread of non-producers is constrained, Rumbaugh *et al.*
 475 ([16, 17]; QS; *P. aeruginosa* in mice), Pollitt *et al.* ([19]; QS, *Staphylococcus*
 476 *aureus* in *G. mellonella*) and Diard *et al.* ([18], T3SS-driven inflammation,
 477 *Salmonella typhimorium* in mice) demonstrated cases where non-producers
 478 spread to high frequencies in host populations. While the reported results were
 479 based on strain frequency counts before and after competition, we here show
 480 that information on social trait expression, temporal infection dynamics and
 481 physical interactions among strains within hosts are essential to understand
 482 whether social traits are important and exploitable in a given system. We thus
 483 posit that more such detailed approaches are required to understand the
 484 importance of bacterial social interactions across host systems and infection
 485 contexts and explain differences between them.

486

487 A deeper understanding of bacterial social interactions inside hosts is
 488 particularly relevant for novel therapeutic approaches that seek to take
 489 advantage of cooperator-cheat dynamics inside hosts to control infections. For
 490 instance, it was proposed that strains deficient for virulence factors could be
 491 introduced into established infections [24]. These strains are expected to
 492 spread because of cheating, thereby reducing the overall virulence factor
 493 availability in the population and the damage to the host. Our results reveal
 494 that virulence-factor-negative strains, although eventually gaining a benefit
 495 from producer strains, are unable to spread in populations. Another
 496 therapeutic approach involves the specific targeting of secreted virulence
 497 factors [25, 27]. This approach is thought to reduce damage to the host and to
 498 compromise resistance evolution [30]. Resistant mutants, resuming virulence
 499 factor production, are not expected to spread because they would act as
 500 cooperators, sharing the benefit of secreted goods with susceptible strains
 501 [76–78]. Our results yet indicate that such resistant mutants could get local
 502 benefits and thus increase to a certain frequency in the population [31]. These
 503 confrontations show that the identification of key parameters driving social
 504 interactions across hosts and infection types is of utmost importance to predict
 505 the success of ‘cheat therapies’ and anti-virulence strategies targeting
 506 secreted public goods.

507

508 **Acknowledgments**

509 We thank two anonymous reviewers for constructive comments and the
 510 Center of Microscopy and Image Analysis (University of Zürich) for support
 511 with image acquisition and advice on image analysis.

512

513 **Funding**

514 This project has received funding from the Swiss National Science Foundation
515 (grant no. PP00P3_165835 and 31003A_182499 to RK and no.
516 P2ZHP3_174751 to EG), and the European Research Council under the grant
517 agreement no. 681295 (to RK).

518

519 **Competing Interests**

520 The authors have no competing interests to declare.

521

522 **Supplementary Information**

523 Supplementary information is available at the journal's website.

524

525 **References:**

- 526 1. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel
527 FM. Common virulence factors for bacterial pathogenicity in plants and
528 animals. *Science* 1995; **268**: 1899–902.
- 529 2. Wu HJ, Wang AHJ, Jennings MP. Discovery of virulence factors of
530 pathogenic bacteria. *Curr Opin Chem Biol* 2008; **12**: 93–101.
- 531 3. Diggle SP, Griffin AS, Campbell GS, West SA. Cooperation and conflict
532 in quorum-sensing bacterial populations. *Nature* 2007; **450**: 411–414.
- 533 4. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA,
534 Kjelleberg S. Biofilms: An emergent form of bacterial life. *Nat Rev*
535 *Microbiol* 2016; **14**: 563–575.
- 536 5. Henkel JS, Baldwin MR, Barbieri JT. Toxins from bacteria. *EXS* 2010;
537 **100**: 1–29.
- 538 6. Granato ET, Harrison F, Kümmerli R, Ross-Gillespie A. Do Bacterial

- 539 “Virulence Factors” Always Increase Virulence? A Meta-Analysis of
540 Pyoverdine Production in *Pseudomonas aeruginosa* As a Test Case.
541 *Front Microbiol* 2016; **7**: 1952.
- 542 7. Köhler T, Buckling A, van Delden C. Cooperation and virulence of
543 clinical *Pseudomonas aeruginosa* populations. *Proc Natl Acad Sci USA*
544 2009; **106**: 6339–6344.
- 545 8. Raymond B, West SA, Griffin AS, Bonsall MB. The dynamics of
546 cooperative bacterial virulence in the field. *Science* 2012; **337**: 85–88.
- 547 9. Harrison F. Bacterial cooperation in the wild and in the clinic: Are
548 pathogen social behaviours relevant outside the laboratory? *BioEssays*
549 2013; **35**: 108–112.
- 550 10. West SA, Diggle SP, Buckling A, Gardner A, Griffin AS. The Social Lives
551 of Microbes. *Annu Rev Ecol Syst* 2007; **38**: 53–77.
- 552 11. Ghoul M, Griffin AS, West SA. Toward an evolutionary definition of
553 cheating. *Evolution* 2014; **68**: 318–331.
- 554 12. Özkaya Ö, Balbontín R, Gordo I, Xavier KB. Cheating on Cheaters
555 Stabilizes Cooperation in *Pseudomonas aeruginosa*. *Curr Biol* 2018; **26**:
556 2070–2080.
- 557 13. Buckling A, Brockhurst MA. Kin selection and the evolution of virulence.
558 *Heredity* 2008; **100**: 484–488.
- 559 14. Leggett HC, Brown SP, Reece SE. War and peace: social interactions in
560 infections. *Philos Trans R Soc Lond B Biol Sci* 2014; **369**: 20130365.
- 561 15. Harrison F, Browning LE, Vos M, Buckling A. Cooperation and virulence
562 in acute *Pseudomonas aeruginosa* infections. *BMC Biol* 2006; **4**: 21.
- 563 16. Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS,
564 West SA. Quorum Sensing and the Social Evolution of Bacterial
565 Virulence. *Curr Biol* 2009; **19**: 341–345.
- 566 17. Rumbaugh KP, Trivedi U, Watters C, Burton-Chellew MN, Diggle SP,
567 West SA. Kin selection, quorum sensing and virulence in pathogenic
568 bacteria. *Proc R Soc B Biol Sci* 2012; **279**: 3584–3588.

- 569 18. Diard M, Garcia V, Maier L, Remus-Emsermann MNP, Regoes RR,
570 Ackermann M, et al. Stabilization of cooperative virulence by the
571 expression of an avirulent phenotype. *Nature* 2013; **494**: 353–356.
- 572 19. Pollitt EJG, West SA, Crusz SA, Burton-Chellew MN, Diggle SP.
573 Cooperation, quorum sensing, and evolution of virulence in
574 *Staphylococcus aureus*. *Infect Immun* 2014; **82**: 1045–1051.
- 575 20. Zhou L, Slamti L, Nielsen-LeRoux C, Lereclus D, Raymond B. The
576 Social Biology of Quorum Sensing in a Naturalistic Host Pathogen
577 System. *Curr Biol* 2014; **24**: 2417–2422.
- 578 21. Harrison F, McNally A, Da Silva AC, Heeb S, Diggle SP. Optimised
579 chronic infection models demonstrate that siderophore ‘cheating’ in
580 *Pseudomonas aeruginosa* is context specific. *ISME J* 2017; **11**: 2492–
581 2509.
- 582 22. Andersen SB, Marvig RL, Molin S, Krogh Johansen H, Griffin AS. Long-
583 term social dynamics drive loss of function in pathogenic bacteria. *Proc*
584 *Natl Acad Sci USA* 2015; **112**: 10756–10761.
- 585 23. Andersen SB, Ghoul M, Marvig RL, Lee Z Bin, Molin S, Johansen HK, et
586 al. Privatisation rescues function following loss of cooperation. *Elife*
587 2018; **7**: e38594.
- 588 24. Brown SP, West S a, Diggle SP, Griffin AS. Social evolution in micro-
589 organisms and a Trojan horse approach to medical intervention
590 strategies. *Philos Trans R Soc Lond B Biol Sci* 2009; **364**: 3157–3168.
- 591 25. Allen RC, Popat R, Diggle SP, Brown SP. Targeting virulence: can we
592 make evolution-proof drugs? *Nat Rev Microbiol* 2014; **12**: 300–308.
- 593 26. Granato ET, Ziegenhain C, Marvig RL, Kümmerli R. Low spatial
594 structure and selection against secreted virulence factors attenuates
595 pathogenicity in *Pseudomonas aeruginosa*. *ISME J* 2018; **12**: 2907–
596 2918.
- 597 27. André JB, Godelle B. Multicellular organization in bacteria as a target for
598 drug therapy. *Ecol Lett* 2005; **8**: 800–810.

- 599 28. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new
600 paradigm for antimicrobial therapy. *Nat Chem Biol* 2007; **3**: 541–548.
- 601 29. Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-
602 mediated disease. *Nat Rev Drug Discov* 2010; **9**: 117–128.
- 603 30. Pepper JW. Drugs that target pathogen public goods are robust against
604 evolved drug resistance. *Evol Appl* 2012; **5**: 757–761.
- 605 31. Rezzoagli C, Wilson D, Weigert M, Wyder S, Kümmerli R. Probing the
606 evolutionary robustness of two repurposed drugs targeting iron uptake in
607 *Pseudomonas aeruginosa*. *Evol Med Public Heal* 2018; **1**: 246–259.
- 608 32. Tan MW, Ausubel FM. *Caenorhabditis elegans*: A model genetic host to
609 study *Pseudomonas aeruginosa* pathogenesis. *Curr Opin Microbiol*
610 2000; **3**: 29–34.
- 611 33. Ewbank JJ. Tackling both sides of the host – pathogen equation with
612 *Caenorhabditis elegans*. *Microbes Infect* 2002; **4**: 247–256.
- 613 34. Papaioannou E, Utari P, Quax W. Choosing an Appropriate Infection
614 Model to Study Quorum Sensing Inhibition in *Pseudomonas Infections*.
615 *Int J Mol Sci* 2013; **14**: 19309–19340.
- 616 35. Félix M-A, Braendle C. The natural history of *Caenorhabditis elegans*.
617 *Curr Biol* 2010; **20**: R965–R969.
- 618 36. Portal-Celhay C, Bradley ER, Blaser MJ. Control of intestinal bacterial
619 proliferation in regulation of lifespan in *Caenorhabditis elegans*. *BMC*
620 *Microbiol* 2012; **12**: 49.
- 621 37. Tan MW, Mahajan-Miklos S, Ausubel FM. Killing of *Caenorhabditis*
622 *elegans* by *Pseudomonas aeruginosa* used to model mammalian
623 bacterial pathogenesis. *Proc Natl Acad Sci USA* 1999; **96**: 715–20.
- 624 38. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ.
625 The multiple signaling systems regulating virulence in *Pseudomonas*
626 *aeruginosa*. *Microbiol Mol Biol Rev* 2012; **76**: 46–65.
- 627 39. Meyer JM, Neely A, Stintzi A, Georges C, Holder IA. Pyoverdinin is
628 essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun* 1996;

- 629 **64**: 518–523.
- 630 40. Takase H, Nitani H, Hoshino K, Otani T. Impact of Siderophore
631 Production on *Pseudomonas aeruginosa* Infections in
632 Immunosuppressed Mice. *Infect Immun* 2000; **68**: 1834–1839.
- 633 41. Cornelis P, Dingemans J. *Pseudomonas aeruginosa* adapts its iron
634 uptake strategies in function of the type of infections. *Front Cell Infect*
635 *Microbiol* 2013; **3**: 1–7.
- 636 42. Smith RS, Iglewski BH. *P. aeruginosa* quorum-sensing systems and
637 virulence. *Curr Opin Microbiol* 2003; **6**: 56–60.
- 638 43. Alibaud L, Köhler T, Coudray A, Prigent-Combaret C, Bergeret E, Perrin
639 J, et al. *Pseudomonas aeruginosa* virulence genes identified in a
640 *Dictyostelium* host model. *Cell Microbiol* 2008; **10**: 729–740.
- 641 44. Lee J, Zhang L. The hierarchy quorum sensing network in
642 *Pseudomonas aeruginosa*. *Protein Cell* 2015; **6**: 26–41.
- 643 45. Zaborin A, Romanowski K, Gerdes S, Holbrook C, Lepine F, Long J, et
644 al. Red death in *Caenorhabditis elegans* caused by *Pseudomonas*
645 *aeruginosa* PAO1. *Proc Natl Acad Sci USA* 2009; **106**: 6327–32.
- 646 46. Kirienko N V., Kirienko DR, Larkins-Ford J, Whlby C, Ruvkun G,
647 Ausubel FM. *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans*
648 iron homeostasis, causing a hypoxic response and death. *Cell Host*
649 *Microbe* 2013; **13**: 406–416.
- 650 47. Cezairliyan B, Vinayavekchin N, Grenfell-Lee D, Yuen GJ, Saghatelian A,
651 Ausubel FM. Identification of *Pseudomonas aeruginosa* phenazines that
652 kill *Caenorhabditis elegans*. *PLOS Pathog* 2013; **9**: e1003101.
- 653 48. Zhu J, Cai X, Harris TL, Gooyit M, Wood M, Lardy M, et al. Disarming
654 *Pseudomonas aeruginosa* Virulence Factor LasB by Leveraging a
655 *Caenorhabditis elegans* Infection Model. *Chem Biol* 2015; **22**: 483–491.
- 656 49. Stiernagle T. Maintenance of *C. elegans*. In: The *C.elegans* Research
657 Community (ed). *WormBook: The Online Review of C. elegans Biology*.
658 2006.

- 659 50. Portman DS. Profiling *C. elegans* gene expression with DNA
660 microarrays. In: The *C.elegans* Research Community (ed). *WormBook:*
661 *The Online Review of C. elegans Biology*. 2006.
- 662 51. Sommer C, Strähle C, Köthe U, Hamprecht FA. ilastik: Interactive
663 Learning and Segmentation Toolkit. *Eighth IEEE Int. Symp. Biomed.*
664 *Imaging (ISBI). Proc.* 2011. pp 230–233.
- 665 52. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch
666 T, et al. Fiji: an open-source platform for biological-image analysis. *Nat*
667 *Methods* 2012; **9**: 676–82.
- 668 53. Preibisch S, Saalfeld S, Tomancak P. Globally optimal stitching of tiled
669 3D microscopic image acquisitions. *Bioinformatics* 2009; **25**: 1463–
670 1465.
- 671 54. Vega NM, Gore J. Stochastic assembly produces heterogeneous
672 communities in the *Caenorhabditis elegans* intestine. *PLOS Biol* 2017;
673 **15**: e2000633.
- 674 55. Ross-Gillespie A, Gardner A, West SA, Griffin AS. Frequency
675 dependence and cooperation: theory and a test with bacteria. *Am Nat*
676 2007; **170**: 331–342.
- 677 56. Kocsis E, Trus BL, Steer CJ, Bisher ME, Steven AC. Image averaging of
678 flexible fibrous macromolecules: the clathrin triskelion has an elastic
679 proximal segment. *J Struct Biol* 1991; **107**: 6–14.
- 680 57. R Development Core Team. R: A language and environment for
681 statistical computing. *R Foundation for Statistical Computing*. 2013.
682 Vienna, Austria.
- 683 58. Dumas Z, Ross-Gillespie A, Kümmerli R. Switching between apparently
684 redundant iron-uptake mechanisms benefits bacteria in changeable
685 environments. *Proc Biol Sci* 2013; **280**: 20131055.
- 686 59. Van Gestel J, Weissing FJ, Kuipers OP, Kovács ÁT. Density of founder
687 cells affects spatial pattern formation and cooperation in *Bacillus subtilis*
688 biofilms. *ISME J* 2014; **8**: 2069–2079.

- 689 60. Weigert M, Kümmerli R. The physical boundaries of public goods
690 cooperation between surface-attached bacterial cells. *Proc R Soc B Biol*
691 *Sci* 2017; **284**: 20170631.
- 692 61. Griffin AS, West SA, Buckling A. Cooperation and competition in
693 pathogenic bacteria. *Nature* 2004; **430**: 1024–1027.
- 694 62. Sandoz KM, Mitzimberg SM, Schuster M. Social cheating in
695 *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci USA*
696 2007; **104**: 15876–81.
- 697 63. Kümmerli R, Griffin AS, West S a, Buckling A, Harrison F. Viscous
698 medium promotes cooperation in the pathogenic bacterium
699 *Pseudomonas aeruginosa*. *Proc Biol Sci* 2009; **276**: 3531–3538.
- 700 64. Popat R, Crusz SA, Messina M, Williams P, West SA, Diggle SP.
701 Quorum-sensing and cheating in bacterial biofilms. *Proc R Soc B Biol*
702 *Sci* 2012; **279**: 4765–4771.
- 703 65. O'Brien S, Luján AM, Paterson S, Cant MA, Buckling A. Adaptation to
704 public goods cheats in *Pseudomonas aeruginosa*. *Proc R Soc B Biol Sci*
705 2017; **284**: 20171089.
- 706 66. van Leeuwen E, O'Neill S, Matthews A, Raymond B. Making pathogens
707 sociable: The emergence of high relatedness through limited host
708 invasibility. *ISME J* 2015; **9**: 2315–2323.
- 709 67. Ross-Gillespie A, Gardner A, Buckling A, West SA, Griffin AS. Density
710 dependence and cooperation: theory and a test with bacteria. *Evolution*
711 2009; **63**: 2315–2325.
- 712 68. Scholz RL, Greenberg EP. Sociality in *Escherichia coli*: Enterochelin Is a
713 Private Good at Low Cell Density and Can Be Shared at High Cell
714 Density. *J Bacteriol* 2015; **197**: 2122–2128.
- 715 69. Pukkila-Worley R, Ausubel FM. Immune defense mechanisms in the
716 *Caenorhabditis elegans* intestinal epithelium. *Curr Opin Immunol* 2012;
717 **24**: 3–9.
- 718 70. Imperi F, Tiburzi F, Visca P. Molecular basis of pyoverdine siderophore

- 719 recycling in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2009;
720 **106**: 20440–20445.
- 721 71. Kümmerli R, Brown SP. Molecular and regulatory properties of a public
722 good shape the evolution of cooperation. *Proc Natl Acad Sci USA* 2010;
723 **107**: 18921–6.
- 724 72. Lindsay RJ, Kershaw MJ, Pawlowska BJ, Talbot NJ, Gudelj I.
725 Harboring public good mutants within a pathogen population can
726 increase both fitness and virulence. *Elife* 2016; **5**: 1–25.
- 727 73. dos Santos M, Ghoul M, West SA. Pleiotropy, cooperation and the social
728 evolution of genetic architecture. *PLOS Biol* 2018; **16**: e2006671.
- 729 74. Ross-Gillespie A, Dumas Z, Kümmerli R. Evolutionary dynamics of
730 interlinked public goods traits: An experimental study of siderophore
731 production in *Pseudomonas aeruginosa*. *J Evol Biol* 2015; **28**: 29–39.
- 732 75. Dandekar AA, Chugani S, Greenberg PE. Bacterial Quorum Sensing
733 and Metabolic Incentives to Cooperate. *Science* 2012; **338**: 264–266.
- 734 76. Mellbye B, Schuster M. The sociomicrobiology of antivirulence drug
735 resistance: a proof of concept. *MBio* 2011; **2**: e00131-11.
- 736 77. Gerdt JP, Blackwell HE. Competition Studies Confirm Two Major
737 Barriers That Can Preclude the Spread of Resistance to Quorum-
738 Sensing Inhibitors in Bacteria. *ACS Chem Biol* 2014; **9**: 2291–2299.
- 739 78. Ross-Gillespie A, Weigert M, Brown SP, Kümmerli R. Gallium-mediated
740 siderophore quenching as an evolutionarily robust antibacterial
741 treatment. *Evol Med Public Heal* 2014; **2014**: 18–29.

742

743 **Figure captions**

744 **Figure 1. Quantifying *P. aeruginosa* infections in the *C. elegans* gut. (A)**

745 Experimental procedure: we used fluorescently tagged *P. aeruginosa* strains
746 to examine bacterial colonization of the *C. elegans* gut. Per experiment, we
747 exposed approximately 200 *C. elegans* nematodes to a lawn of mCherry-
748 tagged PAO1 strains for 24 hours. Subsequently, nematodes were removed
749 from the bacterial plate, surface washed and collected in sterile buffer for
750 monitoring. After 0, 6, or 30 hours post exposure (hpe), approximately 30
751 nematodes were immobilized and transferred to microscopy slides for imaging.

752 (B) Brightfield and fluorescence channel merged image depicting mCherry-
753 fluorescent bacteria inside the host gut. (C) Bacterial load inside the nematode

754 was quantified as the sum of fluorescence intensity across pixels in the region
755 of interest "ROI" (yellow outline) and standardised by total worm area. (D)

756 Colonization dynamics of the wildtype strain PAO1-*mCherry*: immediately after
757 removal from the exposure plate (0 hpe), worms showed high bacterial loads
758 inside their guts. Bacterial load first declined when the worms were kept in
759 buffer for 6 hours, but then remained constant for the next 24 hours. Grey
760 shaded area indicates background fluorescence (mean +/- standard deviation)
761 of worms exposed to the non-fluorescent, non-pathogenic *E.coli* OP50. N =
762 number of worms from four independent experiments. *** = $p < 0.001$, n.s. =
763 not statistically significant.

764

765 **Figure 2. *P. aeruginosa* expresses genes for pyoverdine synthesis and**
766 **quorum sensing regulators in the host gut.** To quantify the expression of

767 virulence factor genes inside hosts, worms were exposed to four PAO1

768 strains, each containing a promoter::*mCherry* fusion for either *pvdA*
 769 (pyoverdine synthesis), *pchEF* (pyochelin synthesis), *lasR* or *rhlR* (quorum
 770 sensing regulators). With the exception of *pchEF*, all genes were significantly
 771 expressed in the host, both at 0 and 30 hpe. Expression levels were
 772 standardised for bacterial load. Grey shaded areas depict background
 773 fluorescence (mean +/- standard deviation) of worms exposed to the non-
 774 fluorescent, non-pathogenic *E.coli* OP50. N = number of worms from four
 775 independent experiments. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not
 776 statistically significant.

777

778 **Figure 3. *P. aeruginosa* can switch between siderophores, while quorum**
 779 **sensing regulators act hierarchically.** Because virulence traits are linked at
 780 the regulatory level, we measured gene expression of each trait in the
 781 negative background of the co-regulated trait. (A) The expression of the
 782 pyoverdine synthetic gene *pvdA* is significantly expressed in the wildtype and
 783 the pyochelin-negative background, but slightly reduced in the latter. (B) The
 784 pyochelin synthetic gene *pchEF* is significantly expressed in the pyoverdine-
 785 negative background, but silent in the wildtype. (C) The expression of the QS-
 786 regulator gene *lasR* is unchanged in the Rhl-negative background compared
 787 to the wildtype. (D) The expression of the QS-regulator gene *rhlR* is reduced in
 788 the Las-negative background. Expression levels were standardised for
 789 bacterial load. Grey shaded areas depict background fluorescence (mean +/-
 790 standard deviation) of worms exposed to the non-fluorescent, non-pathogenic
 791 *E.coli* OP50. N = number of worms from four independent experiments. * = $p <$
 792 0.05 ; ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not statistically significant.

793

794 **Figure 4. Virulence factor production affects bacterial uptake and host**
 795 **colonization ability.** (A) Bacterial load inside *C. elegans* guts measured
 796 immediately after the recovery of worms from the exposure plates (0 hours
 797 post exposure; hpe). Comparisons across isogenic PAO1 mutant strains, each
 798 deficient for the production of one or two virulence factors, reveal that the two
 799 quorum-sensing mutants PAO1 Δ *lasR* and PAO1 Δ *rhIR* reached lower bacterial
 800 densities than the wildtype. (B) Comparison of the relative colonization
 801 success of strains (ratio of bacterial loads at 0 hpe versus 30 hpe) revealed
 802 that the siderophore-negative strain PAO1 Δ *pvdD* Δ *pchEF* showed significantly
 803 reduced ability to remain in the host compared to the wildtype. In contrast, the
 804 colonisation success of PAO1 Δ *pchEF* and PAO1 Δ *rhIR* was increased relative
 805 to the wildtype. Grey shaded areas depict background fluorescence (mean +/-
 806 standard deviation) of worms exposed to the non-fluorescent, non-pathogenic
 807 *E.coli* OP50. N = number of worms from four independent experiments. * = $p <$
 808 0.05; ** = $p < 0.01$, *** = $p < 0.001$.

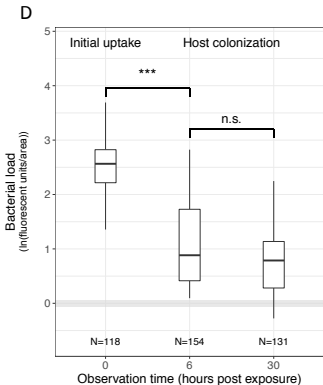
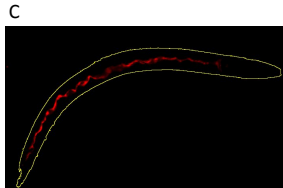
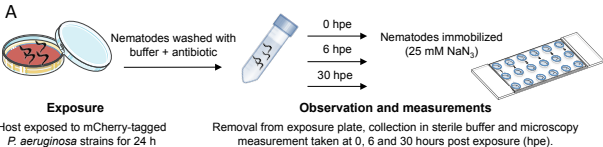
809

810 **Figure 5. Mixed infections reveal social strain dynamics but no**
 811 **successful cheating.** (A) Relative fitness of the wildtype PAO1-*mCherry* after
 812 42 hours of competition inside the *C. elegans* gut against an untagged PAO1
 813 control strain; the siderophore-negative strain PAO1 Δ *pvdD* Δ *pchEF*; and the
 814 Las-negative strain PAO1 Δ *lasR*. The control competition revealed a mild but
 815 significant negative effect of the *mCherry* tag on wildtype fitness. When
 816 accounting for these *mCherry* costs, we found that the putative cheat strains
 817 PAO1 Δ *lasR* and PAO1 Δ *pvdD* Δ *pchEF* performed equally well compared to

the wildtype, but could not outcompete it. This suggests that virulence factor deficient strains benefit from the presence of non-producers but cannot successfully cheat on them. (B) At 6 hpe, wildtype frequency in mixed infections correlated positively with total bacterial load inside hosts in competition with PAO1 Δ lasR (squares and dashed lines) and PAO1 Δ pvdD Δ pchEF (diamonds and dotted lines) but not in the control competition (circles and solid lines). These correlations disappeared at 48 hpe. Each data point represents an individual worm. Data shown in A+B stem from the same three independent experiments.

827

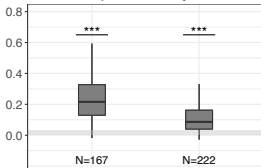
Figure 6. Spatial structure of mixed infections in the nematode gut. (A, B) Illustrative examples of *C. elegans* individuals infected with a mixture of GFP- and mCherry-labelled strains. Each worm was computationally straightened and fluorescence intensity values were extracted for each pixel from tail (X=0) to head (X=1). We then calculated the Spearman correlation coefficient ρ between the intensity values in the two fluorescence channels across pixels, as our estimate of strain colocalization. Examples show worms with high (A) and low (B) degrees of colocalization. (C) Patterns of colocalization levels varied substantially between individuals, but did not differ across strain combinations ($p = 0.119$: wildtype PAO1-*mCherry* versus: (i) wildtype PAO1-*gfp* (circles), (ii) PAO1 Δ pvdD Δ pchEF-*mCherry* (diamonds) or (iii) PAO1 Δ lasR-*mCherry* (squares). Each data point represents an individual worm. Data stems from 3 independent experiments, with 12 replicates each.



Relative promoter expression
(ln(fluorescent units/area))

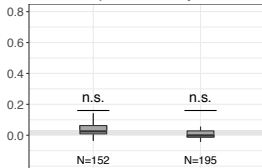
A

pvdA::mCherry



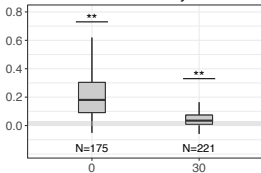
B

pchEF::mCherry



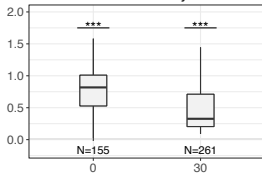
C

lasR::mCherry



D

rhIR::mCherry

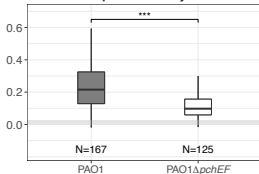


Observation time (hours post exposure)

Relative promoter expression
($\ln(\text{fluorescent units/area})$)

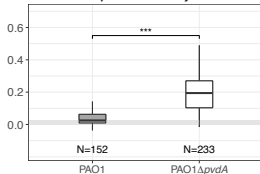
A

pvdA::mCherry



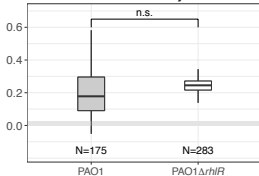
B

pchEF::mCherry



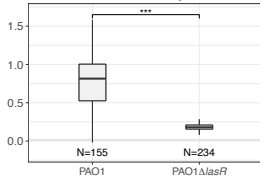
C

lasR::mCherry

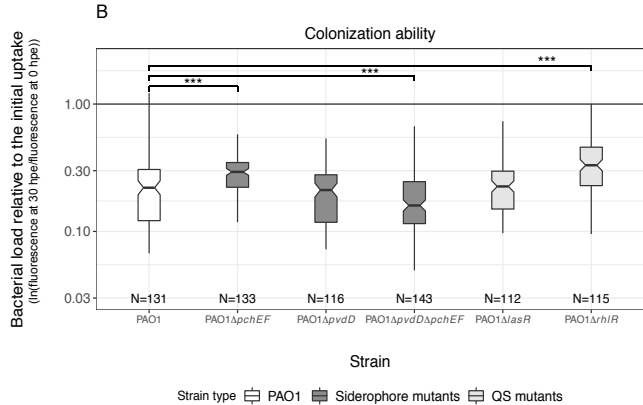
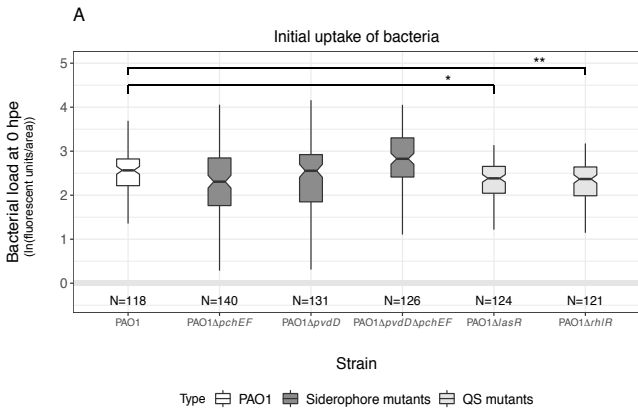


D

rhIR::mCherry

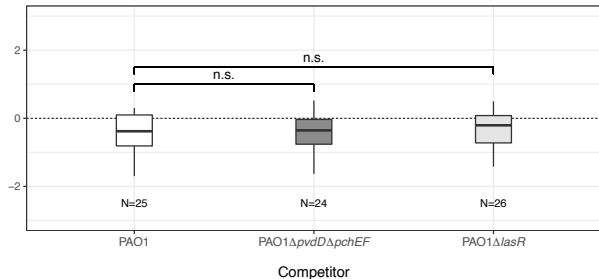


Strain



Fitness of PAO1-*mCherry* (ln(fitness))

A



B

Bacterial load in host gut (CFU)

