

1 **Cheating on orthogonal social traits prevents the tragedy of the commons**  
2 **in *Pseudomonas aeruginosa***

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9 Running Head: Ecology of multiple social traits

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## 13 **Abstract**

14 Bacterial cooperation can be disrupted by non-producers, which can access  
15 public goods without paying their production cost. These cheaters can increase  
16 in frequency, exhausting the public goods and causing a population collapse.  
17 We investigated how interactions among cheaters in orthogonal social traits  
18 influence such collapse. We characterized the dynamics of *Pseudomonas*  
19 *aeruginosa* polymorphic populations under conditions where two social traits,  
20 production of iron-scavenging pyoverdine and quorum sensing regulated  
21 elastase, are necessary. We demonstrate that cheaters for either trait compete  
22 with both the wild type and each other and, since production of pyoverdine is  
23 costlier than elastase production, pyoverdine cheaters impair invasion by  
24 quorum sensing mutants, preventing the collapse caused by the latter. A  
25 mathematical model shows that these dynamics are determined by the costs of  
26 the social traits involved, while their benefits only influence population mean  
27 fitness. Finally, we show how quorum sensing regulation can avoid full loss of  
28 cooperation.

## 29 **Introduction**

30 Although bacteria are unicellular organisms, they can engage in many  
31 group behaviors including biofilm formation, swarming motility, production and  
32 secretion of extracellular proteases and iron-chelating siderophores (1–4). The  
33 collective production of costly, secreted compounds provides fitness benefit to  
34 the entire population and can be considered as cooperative behaviors.  
35 Cooperation is frequently under the threat of exploitation by cheaters:  
36 individuals that benefit from the cooperative action but contribute little or nothing  
37 at all to the production of the public goods. When mixed with cooperators,  
38 cheaters can increase in frequency and cause loss of cooperation by  
39 exhaustion of the public goods, leading to a collapse of the entire population,  
40 characterized by a strong decrease in the growth yield of the entire population  
41 (5). This phenomenon, defined as the ‘tragedy of the commons’, was coined in  
42 economics (6), but has been explored in ecology (7) and has also become a  
43 focus of attention in microbiology in the last decade (8–12). Several  
44 mechanisms have been proposed to explain how cooperative behaviors are still

45 observed and maintained in microbial populations despite the emergence of  
46 cheaters. For instance, spatial structure and diffusion (13–22), pleiotropy (9,  
47 23–30), migration (31), social and non-social adaptations (7, 11, 32, 33),  
48 policing mechanisms (10), molecular properties of public goods (34), and  
49 metabolic strategies (35) play significant roles in maintaining cooperation by  
50 preventing cheater invasions and avoiding the tragedy of the commons (2).  
51 Importantly, despite all these mechanisms to inhibit cheaters' invasion, cheating  
52 behavior is still observed *in vitro* (9–11, 25), *in vivo* (36, 37), and in natural  
53 populations (38–40).

54 Certain cheaters are also clinically relevant and are repeatedly isolated  
55 from the sputum samples of cystic fibrosis (CF) patients chronically infected  
56 with *Pseudomonas aeruginosa* (38, 41–43). CF is a genetic disorder which  
57 causes thickening of mucus in the lungs. Although initial acute infections are  
58 normally associated with colonization of the lungs by wild type (WT) *P.*  
59 *aeruginosa*, subsequent chronic infections consist of polymorphic populations  
60 which include mutants affected in social traits (41, 43–45). Importantly, *in vitro*  
61 studies, which focused on one trait and one constraint at a time, demonstrated  
62 that invasion by a cheater leads to a tragedy of the commons (9–11). However,  
63 despite the prevalence of social cheaters in the CF lung population, population  
64 collapse due to the invasion of cheaters has not been described. Therefore, we  
65 reasoned that studying interactions among multiple social cheaters,  
66 simultaneously, under conditions where more than one social trait is required  
67 could provide new insights into socialdynamics of *P. aeruginosa* populations in  
68 CF lungs and other environments. When more than one environmental  
69 constraint is present, the roles among different social mutants are likely to be  
70 more complex, since a cheater for one trait could potentially be a cooperator for  
71 another, making 'cheater' and 'cooperator' relative terms (46). We hypothesize  
72 that in environments where multiple constraints require bacteria to express  
73 multiple cooperative traits simultaneously, competition among mutants in  
74 orthogonal social traits (traits that are not known to be functionally linked), could  
75 influence their co-existence and the magnitude of the collapse of the population.  
76 This possibility is further supported by recent theoretical and experimental  
77 studies showing that interactions between interlinked cooperative traits  
78 significantly affect the course of their evolution (26, 47).

79 Here we examine the consequences of ecological interactions among  
80 social cheaters and the full cooperators in *P. aeruginosa* populations under  
81 conditions where two orthogonal cooperative traits are required.

82 Both *lasR* and *pvdS* mutants are used individually in a large number of  
83 sociomicrobiology studies (9, 25, 34, 48–52) and are among the most common  
84 mutants recurrently isolated from the sputum samples of CF patients (38, 41,  
85 42). LasR is the master regulator of quorum sensing (QS) and controls the  
86 production of elastase. Production and extracellular secretion of elastase is  
87 essential for *P. aeruginosa* to digest complex sources of amino acids, such as  
88 casein, which serves as carbon and nitrogen source (9). Previous studies  
89 showed that *lasR* mutants grow poorly in media containing casein as the only  
90 carbon source, but increase in frequency when mixed with WT bacteria. This  
91 invasion of the mutant, eventually leads to a collapse where the total cell  
92 numbers of the population are drastically reduced due to the depletion of  
93 producers of the essential public good (9–11). Similarly, production of  
94 pyoverdine is one of the most studied cooperative trait in bacteria (34, 48–52).  
95 In iron-limited environments, pyoverdine is secreted by the *P. aeruginosa*,  
96 chelates iron from the environment and is subsequently retrieved, providing iron  
97 to the cell (49). Mutants in pyoverdine synthesis (e.g. *pvdS*) do not pay the cost  
98 of its production but are still able to retrieve the iron-bound pyoverdine produced  
99 by others, gaining a fitness advantage and increasing in frequency in the  
100 population (34, 53, 54).

101 We followed the cheating behavior of a *lasR* knock-out (KO) mutant in  
102 environments where casein is the sole carbon source, and thus production of  
103 elastase is required. In addition to this ‘one constraint - one trait’ setting, we  
104 added another constraint (iron depletion) and another social player (a *pvdS* KO  
105 mutant) and studied the behavior of the population in a ‘two constraints - two  
106 traits’ setting. We quantified the cheating behavior of a *lasR* mutant in short and  
107 long-term competitions, in iron-supplied or iron-depleted casein media with or  
108 without the presence of a *pvdS* mutant. We found that the relative fitness of the  
109 *lasR* mutant is altered when the *pvdS* mutant is in the culture, but only when the  
110 *lasR* mutant produces pyoverdine. We next determined the long-term  
111 consequences of the interactions among the two mutants and the WT for the  
112 onset of the tragedy of the commons. Our results show that in the environment

113 where the two cooperative traits are required, competition between the two  
114 mutants affects their dynamics, preventing the drastic population collapse  
115 otherwise caused by domination of the *lasR* mutant. Moreover, we developed a  
116 mathematical model which shows that social dynamics in multiple public good  
117 competitions are determined by the differences among the costs of the public  
118 goods involved, while their benefits only affect population mean fitness.

119

## 120 **Results**

### 121 ***Cheating behavior of lasR mutant depends on the environment and the*** 122 ***composition of the population.***

123 We investigated the fitness of *lasR* and *pvdS* mutants, alone and in  
124 competition under different environmental conditions, to determine the effect of  
125 the interactions between different cooperative traits on the dynamics of the  
126 cheater frequency and on the overall fitness of the population. Both mutants  
127 grow as well as WT in media where neither elastase nor pyoverdine are  
128 required (*i.e.*, an iron-supplied medium where casamino acids (CAA) are the  
129 sole carbon source) (Fig. S1A). However, when casein is the sole carbon  
130 source, *lasR* mutant has a lower growth than WT (Fig. S1B) and in iron-  
131 depleted CAA medium the growth yield of *pvdS* mutant is lower than that of WT  
132 and *lasR* mutant (Fig. S1C). Importantly, even though LasR regulates most of  
133 the quorum sensing genes in *P. aeruginosa*, the growth yield of *lasR* mutant  
134 was only affected significantly in media where elastase is required (Fig. 1 and  
135 Fig. S1). These data corroborate that there is no direct functional link between  
136 *lasR* and *pvdS* under the conditions tested (Fig. S1B and S1C) (55, 56). Next,  
137 to obtain a condition where both constraints were present, we cultured these  
138 mutants in a medium with casein as the sole carbon source supplemented with  
139 transferrin to deplete iron (iron-depleted casein medium). Monocultures of both  
140 *lasR* and *pvdS* mutants have a lower growth yield than WT in this medium (Fig.  
141 1) because, under these conditions, elastase and pyoverdine are both required  
142 for growth. Importantly, the growth yield of *lasR* mutant is smaller than that of  
143 *pvdS* mutant.

144 We next determined the relative fitness of these two mutants in  
145 competition with WT. When there is no environmental constraint present,  
146 neither of the mutants show any significant increase in frequency (Fig. S2A).  
147 However, when co-cultured with WT in iron-supplied casein medium, *lasR*  
148 mutant increases in frequency, demonstrating that it can act as cheater under  
149 these conditions (Fig. 2A-left and Fig. S2B). Introduction of the *pvdS* mutant in  
150 the WT:*lasR* co-cultures does not affect the cheating behavior of *lasR* mutant,  
151 since *lasR* can also increase in frequency in the triple co-culture (Fig. 2A-right,  
152 and Fig. S3A). The fact that *pvdS* mutant does not change the behavior of *lasR*  
153 mutant in the iron-supplied casein media is consistent with the fact that *pvdS*  
154 mutant does not increase in frequency, and thus it does not act as a cheater  
155 under these conditions (Fig. 2B and Fig. S3B). Then, we studied the behavior of  
156 these mutants in the medium with two constraints (iron-depleted casein  
157 medium). In this medium, *lasR* mutant again increases in frequency in the co-  
158 cultures with WT (Fig. 2C-left). Importantly, the relative fitness of *lasR* in iron-  
159 depleted casein medium is smaller than the observed in the iron-supplied  
160 casein medium (Fig. 2A-left). This is not due to a differential production of  
161 pyoverdine in *lasR* (Fig. S4) but because, in iron-depleted casein medium, WT  
162 reaches a much smaller growth yield than in iron-supplied casein medium (Fig.  
163 1 and Fig. S1B). In fact, when measured in units of cumulative numbers of cell  
164 divisions (CCD = final cell number – initial cell number) (57, 58) the relative  
165 fitness per cell division of *lasR* is not significantly different in the two media  
166 ( $7.27 \times 10^{-10} \pm 2.19 \times 10^{-10}$  versus  $7.53 \times 10^{-10} \pm 1.20 \times 10^{-10}$  in iron-supplied and iron-  
167 depleted medium, respectively) and thus the relative fitness of the mutants in 48  
168 hours (Fig. 2) is higher in the iron-supplied medium, where the growth yield is  
169 also higher (Fig. 1).

170 Interestingly, when *pvdS* mutant added to the competition in iron-  
171 depleted casein medium, it acts as a cheater in co-cultures with WT (Fig. 2D-  
172 left), and in triple cultures with *lasR* and WT (Fig. 2D-right, and Fig. S3D).  
173 Strikingly, in the triple cultures under the condition where both traits are  
174 required, the presence of *pvdS* mutant results in a significant decrease in the  
175 ability of *lasR* mutant to act as a cheater (Fig. 2C-right, and Fig. S3C). These  
176 results show that the costs and benefits of the two social traits studied here are  
177 context dependent and support the conclusion that the behaviors of the social

178 mutants vary not only with the environment, but also with the level of  
179 polymorphism in the population.

180 ***Cheating capacity of lasR mutant determines the onset of the tragedy of***  
181 ***the commons***

182 We next asked what would be the long-term consequences of these  
183 differences in cheating capacities for the overall fitness of the population by  
184 performing long-term propagations (Fig. 3). We started co-cultures with  
185 WT:*lasR* or WT:*lasR*:*pvdS* (at 9:1 and 8:1:1 initial ratios, respectively), in either  
186 iron-supplied casein medium (Fig. 3A and 3B), or in iron-depleted casein  
187 medium (Fig. 3C and 3D). The illustrations of the four experimental conditions  
188 are shown in Fig. S5. Propagations were performed by culture transfer to fresh  
189 media every 48 hours. Before each passage, cell density and frequencies of  
190 WT, *pvdS*, and *lasR* cells were determined. Growth yields in OD<sub>600</sub> and colony  
191 forming units (CFUs) are shown in Fig. 3 and Fig. S6, respectively.

192 We observed that, in the long-term propagations, in five out of six  
193 replicates of WT:*lasR* co-cultures in iron-supplied casein medium, *lasR* mutant  
194 quickly increased in frequency throughout the first 8 days (4 passages),  
195 reaching up to 90% of the population (red bars in Fig. 3A). The total cell  
196 densities of the populations (black lines) rapidly decreased to levels similar to  
197 that of *lasR* monocultures (OD<sub>600</sub> = 0.03) by day 12, and no recovery was  
198 observed in subsequent passages (Fig. 3A, and Fig. S7). We defined this  
199 density, which was reached by day 12 of the propagation (OD<sub>600</sub> = 0.03), as the  
200 ‘collapse threshold’ caused by the domination of *lasR* mutant. One replicate out  
201 of six did not follow this trend; in this case, no population collapse was  
202 observed, and the total cell numbers remained high throughout the experiment  
203 (Fig. S7B). The cause of this difference is currently under investigation, but the  
204 fact that it only occurred in one of the six replicates suggests that the WT in this  
205 particular replicate may have acquired *de novo* beneficial mutation(s), that could  
206 prevent invasion of *lasR* mutant, and these are likely to be non-social  
207 mutation(s).

208 Next, we analyzed long-term competitions in triple co-culture (WT, *pvdS*,  
209 and *lasR*; respectively 8:1:1) in iron-supplied casein medium (Fig. 3B, and Fig.  
210 S8). In this case, we observed an increase in *lasR* frequency, similar to that of

211 WT and *lasR* co-cultures seen in Fig. 3A, which was also accompanied by a  
212 drastic decrease in the overall population density. At day 10 of the propagation  
213 the six populations reached the collapse threshold. The frequencies of *pvdS*  
214 mutant varied between 4% and 15% throughout the duration of the experiment  
215 with no indication of any sustained increase (blue bars, Fig. 3B). This result is  
216 consistent with the predictions from the relative fitness measurements (Fig. 2B).

217 Then we propagated WT:*lasR* co-cultures in the medium with two  
218 constraints (Fig. 3C, and Fig. S9). In these propagations *lasR* mutant also  
219 increases in frequency throughout the first days, but at a slower pace than in  
220 iron-supplied medium. The total cell numbers remain high until days 10-12, but,  
221 as the *lasR* frequencies increase to about 80%, the density of the population  
222 decreases, reaching the collapse threshold by day 18.

223 Hence, in all the three scenarios described here, the dominance of *lasR*  
224 mutant is followed by a drastic population collapse due to the tragedy of the  
225 commons (Fig. 3 A – C).

### 226 ***pvdS* mutant prevents the drastic population collapse caused by the** 227 ***invasion of lasR* mutant**

228 Our short-term competitions revealed that the cheating ability of *lasR* is  
229 influenced by both abiotic and biotic conditions, as the presence of *pvdS* in the  
230 low iron conditions reduces the relative fitness of *lasR* mutant (Fig. 2C).  
231 Therefore, we investigated if, under low iron conditions, *pvdS* could protect a  
232 polymorphic population from the drastic population collapse caused by *lasR*  
233 invasion. Fig. 3D (and the individual replicates in Fig. S10) shows that in the  
234 propagation of triple co-cultures in iron-depleted casein medium, *lasR* cannot  
235 increase in frequency (it stays at approximately 3% throughout the experiment).  
236 In contrast, *pvdS* rapidly spreads during the first 12 days to an average  
237 frequency of 96% at day 18. Despite the *pvdS* domination, cell densities of the  
238 overall populations stay high. These indicate that, under these conditions, the  
239 presence of only 4% of pyoverdine producers in the population is enough to  
240 sustain the growth of the entire populations to levels similar to the WT mono-  
241 cultures. This interpretation is supported by the results shown in Fig. S11,  
242 representing growth yields of mixed cultures with different starting frequencies  
243 of *pvdS*.

244 Overall, the domination of *pvdS* mutant in the triple cultures with the two  
245 constraints has a remarkable effect on the outcome of the propagations in terms  
246 of the growth yields; *pvdS* domination prevents expansion of *lasR* and thus the  
247 drastic population collapse of the population, which occurs when *lasR* mutant  
248 dominates ( $OD_{600}=0.03$ , Fig. 3A – C). This occurs because, in this environment  
249 where both *lasR* mutant and WT are induced to produce pyoverdine, even  
250 though *lasR* mutant still increases in frequency in relation to the WT (Fig. S3C),  
251 it loses against *pvdS*, given the high relative fitness of *pvdS* against both WT  
252 and *lasR* in this medium (Fig. 2).

253 As a control, we also performed the long-term propagation experiments  
254 in media with no constraints. As expected, we did not observe any significant  
255 change in the population densities (Fig. S12).

256

### 257 ***Manipulation of carbon or iron source availability can prevent or induce*** 258 ***the collapse***

259 We reasoned that if strong ecological interactions dominate in long-term  
260 dynamics over *de novo* adaptive mutations, alterations of the abiotic factors in  
261 the triple cultures should modify the role of each mutant by changing the costs  
262 and benefits of the cooperative traits. Indeed, changing the carbon source from  
263 casein to CAA during the course of the propagation eliminated the behavior of  
264 *lasR* mutant as a cheater, and this environmental change was sufficient to  
265 protect the WT:*lasR* co-cultures from population collapse (Fig. 4A). Conversely,  
266 addition of iron to the iron-depleted casein medium (thus making it iron-  
267 supplied) reverts the expansion of *pvdS* mutant, favoring a consequent increase  
268 in *lasR* cheating capacity, ultimately causing the collapse of all the populations  
269 at day 18 (Fig. 4B). We confirmed that changes in final frequencies observed in  
270 Fig. 4B were not due to the high starting frequencies of *pvdS*; even though the  
271 selective advantage of *pvdS* is frequency dependent, this mutant is capable of  
272 cheating even at frequencies higher than 90% (Fig. S13).

273 Overall, these results show that by changing the roles of *lasR* and *pvdS*  
274 mutants, it was possible to revert the social and ecological dynamics of the  
275 populations in a very predictable and reproducible manner. The different  
276 consequences of these abiotic manipulations are related to the distinct  
277 characteristics of the two mutants studied here, *i. e.*, the tragedy of the

278 commons caused by invasion of *pvdS* causes a small drop in cell density while  
 279 invasion of *lasR* leads to a much greater decrease in density.

280 ***A mathematical model of a 3-way public goods game explains the***  
 281 ***dynamics of the cheating mutants***

282 To further investigate the main general factors determining the dynamics  
 283 of competitions among cooperators and cheaters, we built a simple  
 284 mathematical model assuming that the fate of cooperators and cheaters is  
 285 governed by the costs and the benefits of the cooperative traits. The model  
 286 assumes that the cost ( $c$ ) of a cooperative trait is lower than the benefit ( $b$ )  
 287 associated with this trait ( $b > c > 0$ ). The model also assumes that the benefit  
 288 provided by the cooperative trait is equal for the entire population, as it would be  
 289 in the case of an equally accessible public good in a well-mixed environment.  
 290 Spatial structure, diffusion or privatization, which would alter the benefit gained  
 291 from the public good for cooperators and cheaters asymmetrically, were not  
 292 considered in the model. The parameters used are described in Table 1.

293 **a) Simple 3-way public goods model**

294 We define the fitness of a cooperator and two cheaters mixed in an  
 295 environment where both traits that these mutants cheat on are necessary as:

296 
$$\omega_{coop} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_1 - c_2$$

297 
$$\omega_{ch1} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_2$$

298 
$$\omega_{ch2} = \omega_0 + b_1 (1 - p_{ch1}) + b_2 (1 - p_{ch2}) - c_1$$

299 As can be seen from the fitness definitions of these three players, the  
 300 cheaters always have a higher fitness than the cooperator due to the costs ( $c_1$   
 301 or  $c_2$ ) saved. Assuming a homogeneous environment, and ignoring stochastic  
 302 effects, the population changes according to the replicator equation system:

303 
$$dp_{coop} / dt = p_{coop}(t) (\omega_{coop} - \bar{\omega}) = p_{coop}(t) (-c_1 p_{ch1}(t) - c_2 p_{ch2}(t))$$

304 
$$dp_{ch1} / dt = p_{ch1}(t) (\omega_{ch1} - \bar{\omega}) = p_{ch1}(t) (c_1 (1 - p_{ch1}(t)) - c_2 p_{ch2}(t))$$

305 
$$dp_{ch2} / dt = p_{ch2}(t) (\omega_{ch2} - \bar{\omega}) = p_{ch2}(t) (c_2 (1 - p_{ch2}(t)) - c_1 p_{ch1}(t))$$

306 The change in the mean fitness is given by:

307 
$$\bar{\omega} = \sum_i p_i(t) \omega_i = \omega_0 + p_{coop}(t) \omega_{coop} + p_{ch1}(t) \omega_{ch1} + p_{ch2}(t) \omega_{ch2}$$

308 
$$= \omega_0 + (b_1 - c_1) (1 - p_{ch1}(t)) + (b_2 - c_2) (1 - p_{ch2}(t))$$

309 Fig. 5A shows the predicted mean fitness and final frequencies of the  
310 different strains in the population assuming different  $c_1/c_2$  ratios. It can be easily  
311 seen that cooperators will always go extinct, and that the two cheaters can only  
312 co-exist when  $c_1 = c_2$ .

313 Whenever  $c_1 \neq c_2$ , then the cheater that produces the more costly trait will  
314 lose. Therefore, the relation between  $c_1$  and  $c_2$  determines which cheater will  
315 dominate the population, independently of the benefits of these cooperative  
316 traits. On the other hand, the yield of the population will depend on,  $\bar{\omega}$ , which is  
317 affected by the difference between  $b$  and  $c$  values of each trait.

318 We simulated the four scenarios (Fig. S14 A – D) corresponding to the  
319 conditions in Fig. 3. In Fig. S14A and S14C, the cooperator for both traits (WT)  
320 and the cheater of the 1<sup>st</sup> cooperative trait compete ( $p_{coop}(0) = 0.9$  and  $p_{ch1}(0) =$   
321  $0.1$ ), while the cheater of the 2<sup>nd</sup> cooperative trait is absent (hence  $p_{ch2}(0)=0$ ).  
322 Whereas, in Fig. S14B and Fig. S14D all three strains compete ( $p_{coop}(0) = 0.8$   
323 and  $p_{ch1}(0) = p_{ch2}(0) = 0.1$ ). In Fig. S14A and S14B, only the 1<sup>st</sup> cooperative trait  
324 is produced ( $b_1 > c_1 > 0$ , whereas  $b_2 = c_2 = 0$ ) while in S14C and S14D both  
325 traits are expressed ( $c_2 > c_1 > 0$  and  $b_1 > b_2 > 0$ ). The  $c_1/c_2$  ratio reflects the  
326 ratios of the relative fitnesses in Fig. 2C and 2D. The time scale of the  
327 simulations reflects the CCD as a more meaningful scale than days or numbers  
328 of generations (57, 58). CCD values were chosen according to the final number  
329 of cells measured in the competition in Fig. 2, where the CCD were significantly  
330 higher in iron-supplied casein medium than in iron-depleted casein medium for  
331 the same time period.  $\omega_0$ ,  $b_1$ , and  $b_2$  are chosen to reflect the growth yields in  
332 Fig. 1 and Fig. S1B.

333 The results of the model for the four scenarios (Fig. S14 A–D) resemble  
334 the experimental data, explaining changes in frequencies and the mean fitness  
335 observed reasonably well. However, this simple model always predicts  
336 complete fixation of the winning mutant (Fig. S14 A–D), and cannot explain the  
337 lack of fixation of the mutants observed experimentally with *lasR* (in Fig. 3 A–C)  
338 and *pvdS* (Fig. 3D). We experimentally tested whether fixation of the winning  
339 mutant could occur if the propagations were continued. Our results show that,  
340 *pvdS* mutant can reach fixation when co-cultured either with WT, or with WT  
341 and *lasR* mutant (Fig. S15B and Fig. S15E, respectively). However, *lasR*  
342 mutant fails to reach fixation (Fig. 6A). In the absence of *pvdS*, when we initiate

343 competitions with initial *lasR* frequencies similar to those at day 18 in Fig. 3A,  
344 *lasR* mutant fails to reach fixation and its frequencies stay around the levels  
345 similar to the ones observed in Fig. 3A after the populations collapse.

### 346 **b) Simple 3-way public goods model including quorum sensing**

347 Given that the *lasR* gene and elastase production are regulated via  
348 quorum sensing, we hypothesized that QS could be responsible for the lack of  
349 fixation of *lasR* mutant. QS regulation should reduce both the cost and the  
350 benefit of elastase production when the cooperators are below the QS threshold  
351 as the cells will not be producing it. We therefore modelled the effect of QS on  
352 fitness equations by assuming a Hill function where the cost ( $c_1$ ) and benefit ( $b_1$ )  
353 of the 1<sup>st</sup> cooperative trait are sharply reduced when the frequency the cheater  
354 of the 1<sup>st</sup> cooperative trait ( $p_{ch1}$ ) reaches a given threshold value ( $th$ ), as:

$$355 \omega_{coop} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_1 (1 / (1 + (p_{ch1} / th)^n)) - c_2$$

$$356 \omega_{ch1} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_2$$

$$357 \omega_{ch2} = \omega_0 + b_1 (1 - p_{ch1}) (1 / (1 + (p_{ch1} / th)^n)) + b_2 (1 - p_{ch2}) - c_1 (1 / (1 + (p_{ch1} / th)^n))$$

358 The change in the mean fitness is given by:

$$359 \bar{\omega} = \omega_0 + ((b_1 - c_1) (1 - p_{ch1}(t)) / (1 / (1 + (p_{ch1} / th)^n))) + (b_2 - c_2) (1 - p_{ch2}(t))$$

360 In this case, fixation of one mutant can only happen if  $c_1 < c_2$ . When  $c_1 \geq c_2$ ,  
361 both cheaters can co-exist in the population (Fig. 5B).

362 As shown in Fig. 7, the results of the simulations of the modified model  
363 including QS for the four experimental conditions predict accurately both the  
364 frequency dynamics and the reduction in population size (assumed to be related  
365 to the mean fitness) as the cheaters spread. It is also now clear that by adding  
366 the QS regulation to the model the simulation predicts that *lasR* will not reach  
367 fixation.

368 To test experimentally if QS regulation prevents fixation of *lasR* in the  
369 WT:*lasR* competitions, we repeated the propagation experiment, shown in Fig.  
370 6A, with the addition of the QS autoinducer AHLs (3OC<sub>12</sub>-HSL) to the culture  
371 medium. Addition of AHLs abolishes the QS-dependent regulation of elastase  
372 by locking the LasR regulator on its ON state. With the addition of AHLs,

373 frequency of *lasR* mutant increases throughout the competitions until fixation  
374 (Fig. 6B), just like the model without QS had predicted (Fig. S14 A – C).

375 In supplementary material, we present other scenarios predicted by this  
376 model, which can be tested experimentally in the future (Fig. S16 – 19).

377 We conclude that quorum sensing regulation of production of a public  
378 good prevents full domination of the QS cheater, maintaining cooperation in  
379 populations. However, if the cheater which wins is affected in the production of  
380 a public good that is not regulated via QS (e.g. *pvdS*) this mutant can dominate  
381 the entire population. In summary, the results obtained from our mathematical  
382 model (Fig. 7) show that the dynamics observed in our propagation experiments  
383 in Fig. 3 can be explained by the relationship between the cost values of two  
384 orthogonal cooperative traits and a quorum threshold that regulates both costs  
385 and benefits of one of these traits.

386

## 387 **Discussion**

388 The classical experimental approach in sociomicrobiology has been to  
389 study one trait and one constraint at a time. The simplicity of such an approach  
390 has substantially increased our understanding of the dynamics of cooperative  
391 and non-cooperative clones and revealed several mechanisms involved in the  
392 maintenance of cooperation (2, 4). The ability of *lasR* or *pvdS* mutants to  
393 behave as cheaters is well documented under these ‘one constraint-one trait’  
394 laboratory settings. However, even though *lasR* and *pvdS* mutants are  
395 commonly isolated from bacterial populations colonizing CF lungs, population  
396 collapse due to the invasion by these mutants has not been seen in patients  
397 (38, 41).

398 Here, we established an experimental setup where WT cooperates in  
399 more than one trait: production of elastase and pyoverdine. Under this  
400 environment with two constraints, the *lasR* mutant is a cheater for elastase but,  
401 a cooperator for pyoverdine, whereas *pvdS* mutant does the opposite. In this  
402 environment, the advantage of *pvdS* mutant for not producing pyoverdine is  
403 higher than that of *lasR* for not producing elastase (Fig. 2C and Fig. 2D). As a  
404 consequence of the different costs associated with the different traits, in 3-way

405 competitions, *pvdS* causes a reduction in the relative fitness of *lasR* mutant and  
406 dominates the population. This domination of *pvdS* prevents the population from  
407 a potential drastic collapse caused by invasion of *lasR* mutants (compare Fig.  
408 3D with Fig. 3 A – C). Although the tragedy of the commons due domination of  
409 *pvdS* mutant can also occur (Fig. S15), the consequent decrease in cell density,  
410 is much less drastic than the decrease in growth yields observed upon  
411 domination of *lasR* mutant (Fig. 1 and Fig. 3 A – C). This happens because the  
412 difference between the benefit and the cost of pyoverdine production is much  
413 smaller than that of elastase production.

414         The results from the 3-way competition demonstrate that by having more  
415 than one environmental constraint and more than one social mutant, a scenario  
416 likely to be closer to the conditions in nature (such as in the lungs of CF  
417 patients), the cheater for the trait with the highest cost is expected to dominate.  
418 The consequence of that domination for the population collapse will depend on  
419 the benefit-cost that such trait entails. Importantly, the degree of the population  
420 collapse caused by the tragedy of the commons can eventually have very  
421 different consequences for the host. In case of a trait whose difference in  
422 benefit to cost is high a drastic collapse on the density of the population caused  
423 by the cheater in that trait is expected. If drastic collapse in density takes place,  
424 clearance of the pathogen is more likely to occur, resulting in a higher benefit to  
425 the host. In contrast, if the mutant for the trait with a low benefit to cost  
426 difference (as for *pvdS*) wins, a weak collapse occurs, to the detriment of the  
427 host.

428         Altogether, our results provide support for a dynamic view of cooperation  
429 and cheating that is dependent on the genotypes and constraints present in the  
430 environment. We demonstrate how changes in the abiotic environment can  
431 make a social mutant to stop cheating on one trait while still cooperating on  
432 other traits also susceptible to cheating. Moreover, both the mathematical  
433 model and experimental results highlight the importance of the difference  
434 between costs of the difference traits for the population dynamics, and the  
435 difference between benefits and costs of each trait for the mean fitness of the  
436 population. Given the relationship between the costs, in a fluctuating  
437 environment, polymorphism of various mutants of cooperative traits, as it occurs  
438 in CF infections, is possible. Additionally, as shown here for *lasR* mutant,

439 quorum sensing regulation can also favor the maintenance of polymorphism, as  
440 such regulation alters the values of the cost and benefits of the traits.

441 A better understanding of the interactions in polymorphic bacterial  
442 populations in complex environments not only provides insights into key aspects  
443 of sociomicrobiology, but also can provide a theoretical framework for the  
444 development of new therapeutic strategies against bacterial populations where  
445 social mutants can invade. *P. aeruginosa* has been the focus of many clinical  
446 CF studies because *lasR* and *pvdS* mutants are repeatedly observed in the  
447 lungs of chronically infected patients (38, 41). Recently, it was suggested that  
448 controlled introduction of engineered *lasR* or *pvdS* cheaters into the lungs of CF  
449 patients infected by *P. aeruginosa* might decrease the bacterial population by  
450 inducing a tragedy of the commons (36, 59, 60). However, in the lungs, *P.*  
451 *aeruginosa* faces multiple constraints similar to the ones studied here: complex  
452 carbon sources and low iron concentrations. Thus, the order of the introduction  
453 of the cheaters, the composition of the bacterial population at the onset of the  
454 intervention, and the abiotic environmental conditions in the CF lungs are  
455 determinants for the ecological outcome of the population and the success of  
456 the intervention. For example, according to our results, introducing a *pvdS*  
457 mutant into a population containing *lasR* mutants in iron-limiting conditions  
458 might avert a drastic population collapse rather than triggering it. A study on the  
459 evolution of *P. aeruginosa* strains in the CF lungs showed that appearance of  
460 *lasR* mutations is followed by that of *pvdS* mutations (and other mutation  
461 affecting in iron metabolism) (41). This might explain why a drastic population  
462 collapse does not take place in the CF lungs. Our results suggest that a  
463 successful clearance of a *P. aeruginosa* infection in CF patients via triggering a  
464 tragedy of the commons can be achieved if the cheaters are introduced when  
465 the environmental constraints are limited to the specific trait that the cheater  
466 strain cheats on. Importantly, modifications of the environmental conditions can  
467 contribute to this effect. For instance, our results predict that when a *lasR*  
468 mutant is introduced, the supply of extra iron could accelerate the drastic  
469 collapse caused by the expansion of *lasR* mutant (Fig. 4B) and presumably  
470 addition of AHLs, by forcing WT to constitutively cooperate, can promote  
471 complete fixation of *lasR* mutants (Fig. 6B), therefore increasing the efficacy of  
472 this potential treatment. Our studies, do not take into account the effect of *pvdS*,

473 *lasR* double mutant (we found no evidence for emergence of double mutants  
474 within the period of our experiments), which have the potential to occur *in vivo*  
475 (41). The effect of such double mutants should be investigated in the future.  
476 However, based on our results, we can speculate that double mutants should  
477 accelerate the collapse of the population. Indeed, we have preliminary data  
478 (data not shown) that in longer propagation experiment (in Fig. 3D), *de novo*  
479 mutations can occur, which include *pvdS*, *lasR* double mutants. We further note  
480 that, while our *pvdS* mutant does not show any advantage in iron-supplied  
481 medium (Fig. 2B), some pyoverdine mutants in *Pseudomonas fluorescens* have  
482 been reported to be better adapted even in environments where iron  
483 concentration is not low and thus can be considered non-social mutations (46).  
484 The observation of these potentially non-social mutants indicates that iron-  
485 supplementation may sometimes fail as potential intervention for clearance,  
486 highlighting the need for understanding the nature of these mutations and  
487 changes in dynamics caused by them.

488 Collectively, our findings underline the need for including polymorphism  
489 and multiple constraints in experimental studies and mathematical models  
490 pertaining to cooperation in microbial populations. Our results demonstrate that  
491 using experimental conditions that include more than one social trait can reveal  
492 complex and dynamic social roles in bacterial populations as well as their  
493 dependence on the environment. Understanding the dynamics of polymorphic  
494 populations in these complex environments provides insights into social  
495 interaction processes, expanding their relevance beyond sociomicrobiology, in  
496 addition to providing knowledge important for the development of new  
497 therapeutic tools.

498

## 499 **Materials and Methods**

500 **Bacterial strains, media and culture conditions.** We used *Pseudomonas*  
501 *aeruginosa* WT strain PAO1 and its isogenic derivatives harboring a gentamycin  
502 resistant gene inserted in either *pvdS* (*pvdS::Gm*) or *lasR* (*lasR::Gm*) (61). Iron-  
503 supplied casein medium contains casein (Sigma, Ref: C8654) (1% w/v) as the  
504 sole carbon and nitrogen sources salts (1.18 g  $K_2HPO_4 \cdot 3H_2O$  and 0.25 g

505 MgSO<sub>4</sub>·7H<sub>2</sub>O per liter of dH<sub>2</sub>O) and 50 μM of FeCl<sub>3</sub>. Iron-depleted casein  
506 medium is identical to the iron-supplied casein medium but instead of FeCl<sub>3</sub>  
507 supplementation, this medium contains 100 μg/ml of human apo-transferrin  
508 (Sigma, T2036) and 20 mM sodium bicarbonate to deplete available iron and  
509 induce pyoverdine production. The medium with no constraints contains the  
510 same salt solutions as the other media, low iron CAA (BD, Ref: 223050) (1%  
511 w/v) as the sole carbon source and 50 μM of FeCl<sub>3</sub>. All cultures were incubated  
512 at 37°C with aeration (240 rpm, New Brunswick E25/E25R Shaker) for the  
513 incubation times indicated. Cell densities were estimated by measuring  
514 absorbance (Abs) at 600 nm (OD<sub>600</sub>) in a Thermo Spectronic Helios δ  
515 spectrophotometer.

516 **Determination of genotypic frequencies.** Estimation of the frequencies of  
517 each strain in the co-cultures was performed by scoring fluorescence and  
518 colony morphology of colonies obtained from plating serial PBS dilutions of the  
519 cultures. For each individual sample, three aliquots (of 50μl - 200μl, as  
520 appropriated) were plated into LB agar plates, which were used as technical  
521 replicates. Then, CFU/ml were calculated by scoring different colony  
522 morphologies on each plates. A stereoscope (Zeiss Stereo Lumar V12) with a  
523 CFP filter was used to distinguish pyoverdine producers, which are fluorescent,  
524 from the non-fluorescent *pvdS* mutants (62, 63). *lasR* mutant colonies have  
525 distinct colony morphology: smaller with smooth edges whereas elastase  
526 producers are larger with rugged edges (63). To validate the phenotypic scoring  
527 all colonies used to determine the frequency from day 18 of the propagation  
528 experiments (Fig. 3D) were tested by PCR with primers for the *lasR* and *pvdS*  
529 genes. The PCR data confirmed the phenotypic scoring with 100% accuracy.

530 **Competition experiments.** We propagated six replicates under four different  
531 conditions (Fig. 3). Prior to start the competition experiments, all strains were  
532 inoculated, from frozen stocks, in medium containing 1% (w/v) casein and 1%  
533 (w/v) CAA in salts solution (same as in iron-supplied casein medium, described  
534 above) for 36 hours at 37°C temperature with shaking (240 rpm). Cells were  
535 then washed with PBS four times, to remove any residual extracellular factor.  
536 Next after measuring cell densities (OD<sub>600</sub>), cultures were normalized to OD<sub>600</sub> =  
537 1.0 and used to inoculate the various media as described in the text and figures.

538 The different strains were diluted into fresh media, at different ratios as  
539 specified, to a starting initial  $OD_{600} = 0.05$ . For short term competitions the  
540 relative frequencies were determined by plating an aliquot of each culture at the  
541 beginning of the experiment ( $t = 0$ ), and after 48 hours of incubation. For long-  
542 term competitions, the relative frequencies were determined at  $t = 0$ , and  
543 thereafter every 48 hours before each passage. At the end of every 48 hours  
544 1.5  $\mu$ l of each culture was transferred to 1.5ml of fresh medium (bottle-neck of  
545 1/1000).

546 **Statistical analysis.** Independent biological replicates were separately grown  
547 from the frozen stocks of each strain. Each experiment was performed at least  
548 twice, with three biological replicates, except in Fig. 2A where one experiment  
549 has only two biological replicates. Each figure (or figure panel) includes data  
550 from the biological replicates of at least two experiments. The sample size (N),  
551 corresponds to the total numbers of independent biological replicates in each  
552 figure panel and is provided in the corresponding figure legends. Relative  
553 fitness was used to determine the cheating capacity of each mutant. For both  
554 *lasR* and *pvdS* strains, the relative fitness ( $\omega$ ) was calculated as the frequency  
555 change over 48 hours relative to the rest of the strains in the mixture, using the  
556 following formula  $\omega = f_{\text{final}} (1 - f_{\text{initial}}) / f_{\text{initial}} (1 - f_{\text{final}})$  where  $f_{\text{initial}}$  is the mean of the  
557 initial proportion measured (as described above) at the beginning of the  
558 competitions while  $f_{\text{final}}$  is the mean of the final proportions of the mutant after 48  
559 hours of competition (62, 63). We used Mann-Whitney test which is a non-  
560 parametric test that does not account for normality and it is more suitable for the  
561 sample size used in each experiment ( $5 < N < 20$ ). For multiple corrections, we  
562 used Kruskal-Wallis test with Dunn's correction. For all statistical analyses we  
563 used GraphPad Prism 6 software ([http://www.graphpad.com/scientific-](http://www.graphpad.com/scientific-software/prism)  
564 [software/prism](http://www.graphpad.com/scientific-software/prism)).

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## 577 **References**

- 578 1. Foster KR (2010) Social behaviour in microorganisms. *Social Behaviour:  
579 Genes, Ecology and Evolution*, eds Székely T, Moore AJ and Komdeur J,  
580 (Cambridge University Press, Cambridge), pp 331–356.
- 581 2. Bruger E, Waters C (2015) Sharing the sandbox: Evolutionary  
582 mechanisms that maintain bacterial cooperation. *F1000Research* 4(0):2–  
583 9.
- 584 3. Parsek MR, Greenberg EP (2005) Sociomicrobiology: The connections  
585 between quorum sensing and biofilms. *Trends Microbiol* 13(1):27–33.
- 586 4. Xavier JB (2016) Sociomicrobiology and Pathogenic Bacteria. *Microbiol  
587 Spectr* 4(3)(June):1–10.
- 588 5. Rankin DJ, Bargum K, Kokko H (2007) The tragedy of the commons in  
589 evolutionary biology. *Trends Ecol Evol* 22(12):643–651.
- 590 6. Hardin G (1968) The Tragedy of the Commons. *Science* 162(3859):1243–  
591 1248.
- 592 7. Waite AJ, Shou WY (2012) Adaptation to a new environment allows  
593 cooperators to purge cheaters stochastically. *Proc Natl Acad Sci U S A*  
594 109(47):19079–19086.
- 595 8. MacLean RC (2008) The tragedy of the commons in microbial  
596 populations: insights from theoretical, comparative and experimental  
597 studies. *Heredity (Edinb)* 100(3):471–477.
- 598 9. Dandekar AA, Chugani S, Greenberg EP (2012) Bacterial Quorum  
599 Sensing and Metabolic Incentives to Cooperate. *Science* 338(6104):264–  
600 266.
- 601 10. Wang M, Schaefer AL, Dandekar AA, Greenberg EP (2015) Quorum  
602 sensing and policing of *Pseudomonas aeruginosa* social cheaters. *Proc*

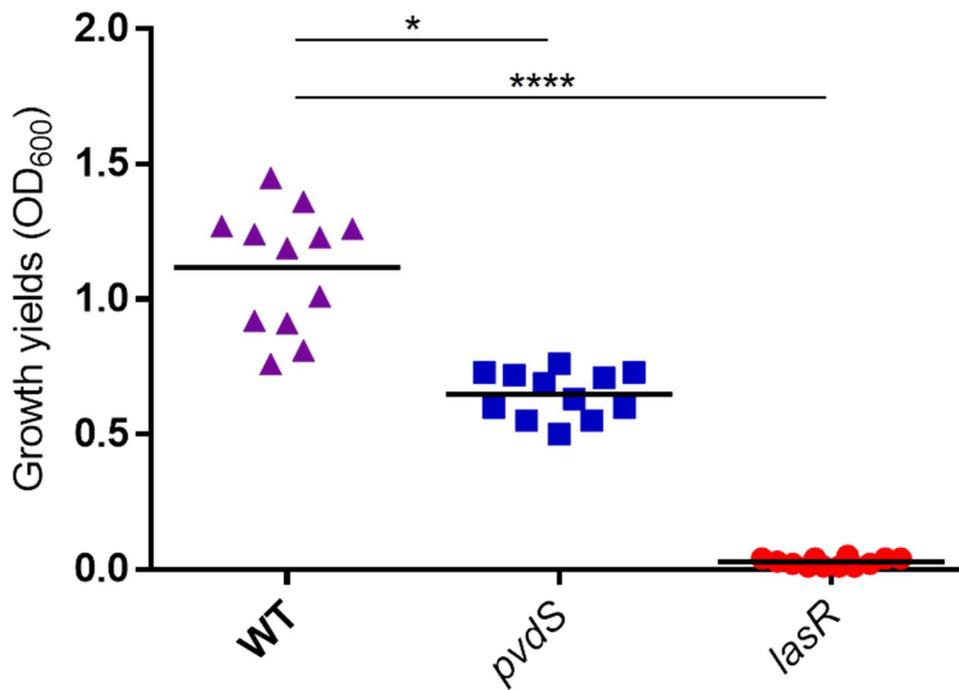
- 603           *Natl Acad Sci* 112(7):2187–2191.
- 604 11. Asfahl KL, Walsh J, Gilbert K, Schuster M (2015) Non-social adaptation  
605 defers a tragedy of the commons in *Pseudomonas aeruginosa* quorum  
606 sensing. *ISME J* 9(8):1734–1746.
- 607 12. West SA, Griffin AS, Gardner A, Diggle SP (2006) Social evolution theory  
608 for microorganisms. *NatRevMicrobiol* 4(1740–1526):597–607.
- 609 13. Krakauer DC, Pagel M (1995) Spatial Structure and the Evolution of  
610 Honest Cost-Free Signalling. *Proc R Soc B Biol Sci* 260(1359):365–372.
- 611 14. Foster KR, Shaulsky G, Strassmann JE, Queller DC, Thompson CRL  
612 (2004) Pleiotropy as a mechanism to stabilize cooperation. *Nature*  
613 431(7009):693–6.
- 614 15. Kreft J-U (2004) Biofilms promote altruism. *Microbiology* 150(8):2751–  
615 2760.
- 616 16. Lion S, Baalen M Van (2008) Self-structuring in spatial evolutionary  
617 ecology. *Ecol Lett* 11(3):277–295.
- 618 17. Kümmerli R, Griffin AS, West S a, Buckling A, Harrison F (2009) Viscous  
619 medium promotes cooperation in the pathogenic bacterium *Pseudomonas*  
620 *aeruginosa*. *Proc Biol Sci* 276(1672):3531–3538.
- 621 18. Nadell CD, Foster KR, Xavier JB (2010) Emergence of spatial structure in  
622 cell groups and the evolution of cooperation. *PLoS Comput Biol*  
623 6(3):e1000716.
- 624 19. West SA, Winzer K, Gardner A, Diggle SP (2012) Quorum sensing and  
625 the confusion about diffusion. *Trends Microbiol* 20(12):586–594.
- 626 20. Dobay A, Bagheri HC, Messina A, Kümmerli R, Rankin DJ (2014)  
627 Interaction effects of cell diffusion, cell density and public goods  
628 properties on the evolution of cooperation in digital microbes. *J Evol Biol*  
629 27(9):1869–1877.
- 630 21. Drescher K, Nadell CD, Stone HA, Wingreen NS, Bassler BL (2014)  
631 Solutions to the public goods dilemma in bacterial biofilms. *Curr Biol*  
632 24(1):50–55.
- 633 22. Persat A, et al. (2015) The mechanical world of bacteria. *Cell* 161(5):988–  
634 997.
- 635 23. Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas*

- 636            *aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A* 102(31):11076–  
637            81.
- 638 24.    Harrison F, Buckling A (2009) Siderophore production and biofilm  
639            formation as linked social traits. *ISME J* 3(5):632–634.
- 640 25.    Sandoz KM, Mitzimberg SM, Schuster M (2007) Social cheating in  
641            *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci U S A*  
642            104(40):15876–15881.
- 643 26.    Ross-Gillespie A, Dumas Z, Kümmerli R (2015) Evolutionary dynamics of  
644            interlinked public goods traits: An experimental study of siderophore  
645            production in *Pseudomonas aeruginosa*. *J Evol Biol* 28(1):29–39.
- 646 27.    Wilder CN, Diggle SP, Schuster M (2011) Cooperation and cheating in  
647            *Pseudomonas aeruginosa*: the roles of the *las*, *rhl* and *pqs* quorum-  
648            sensing systems. *ISME J* 5(8):1332–43.
- 649 28.    Friman V-P, Diggle SP, Buckling A (2013) Protist predation can favour  
650            cooperation within bacterial species. *Biol Lett* 9(5):20130548.
- 651 29.    Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003)  
652            Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing  
653            Regulons : Effects of Growth Phase and Environment. *J Bacteriol*  
654            185(7):2080–2095.
- 655 30.    Bachmann H, et al. (2013) Availability of public goods shapes the  
656            evolution of competing metabolic strategies. *Proc Natl Acad Sci U S A*  
657            110(35):14302–7.
- 658 31.    Kerr B, Neuhauser C, Bohannan BJM, Dean AM (2006) Local migration  
659            promotes competitive restraint in a host–pathogen “tragedy of the  
660            commons.” *Nature* 442(7098):75–78.
- 661 32.    Hammarlund SP, Connelly BD, Dickinson KJ, Kerr B (2016) The evolution  
662            of cooperation by the Hankshaw effect. *Evolution (N Y)* 70(6):1376–1385.
- 663 33.    Kümmerli R, et al. (2015) Co-evolutionary dynamics between public good  
664            producers and cheats in the bacterium *Pseudomonas aeruginosa*. *J Evol*  
665            *Biol* 28(12):2264–2274.
- 666 34.    Kümmerli R, Brown SP (2010) Molecular and regulatory properties of a  
667            public good shape the evolution of cooperation. *Proc Natl Acad Sci U S A*  
668            107(44):18921–6.

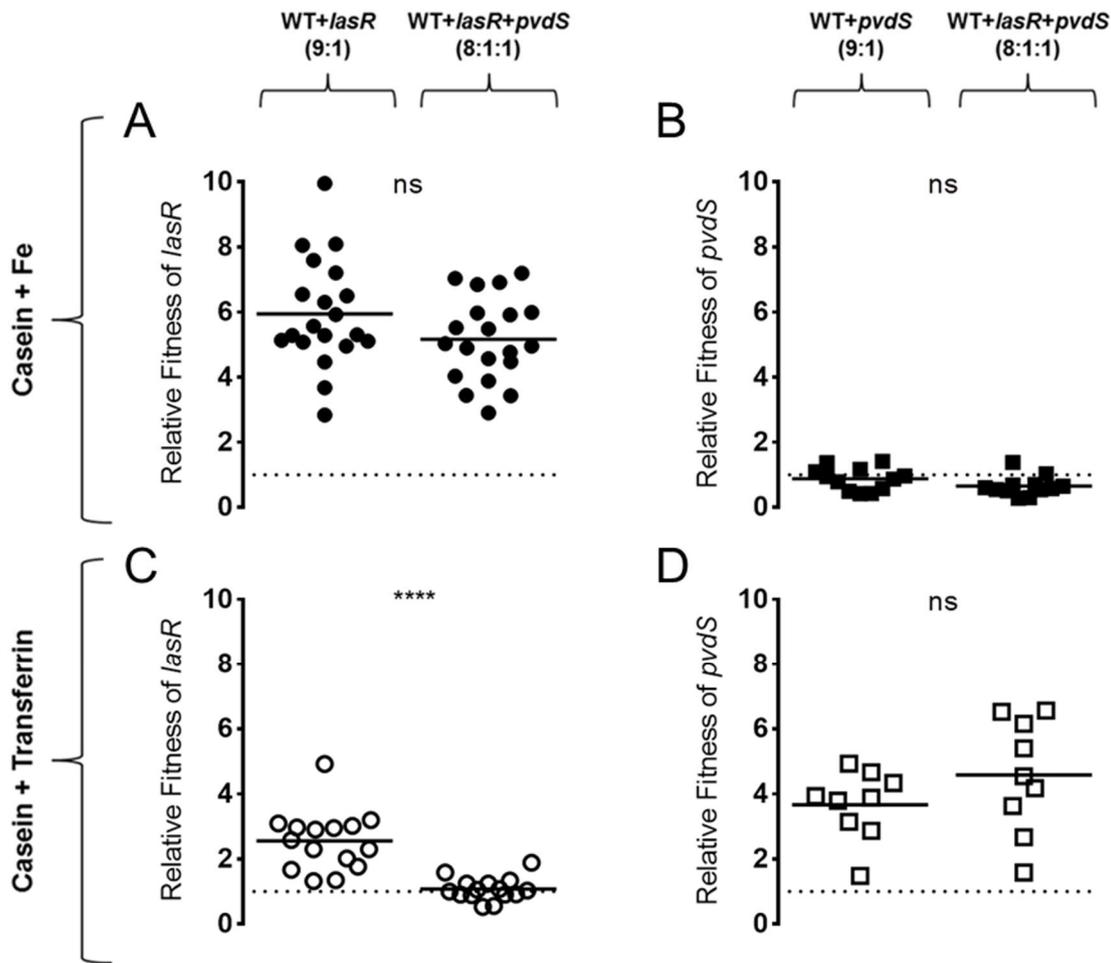
- 669 35. Xavier JB, Kim W, Foster KR (2011) A molecular mechanism that  
670 stabilizes cooperative secretions in *Pseudomonas aeruginosa*. *Mol*  
671 *Microbiol* 79(1):166–179.
- 672 36. Rumbaugh KP, et al. (2009) Quorum Sensing and the Social Evolution of  
673 Bacterial Virulence. *Curr Biol* 19(4):341–345.
- 674 37. Czechowska K, McKeithen-Mead S, Al Moussawi K, Kazmierczak BI  
675 (2014) Cheating by type 3 secretion system-negative *Pseudomonas*  
676 *aeruginosa* during pulmonary infection. *Proc Natl Acad Sci U S A*  
677 111(21):7801–7806.
- 678 38. Winstanley C, O'Brien S, Brockhurst MA (2016) *Pseudomonas*  
679 *aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis  
680 Chronic Lung Infections. *Trends Microbiol* 24(5):327–337.
- 681 39. Cordero OX, Polz MF (2014) Explaining microbial genomic diversity in  
682 light of evolutionary ecology. *Nat Rev Microbiol* 12(4):263–273.
- 683 40. Katzianer DS, Wang H, Carey RM, Zhu J (2015) Quorum non-sensing:  
684 social cheating and deception in *Vibrio cholerae*. *Appl Environ Microbiol*  
685 81(11):3856–3862.
- 686 41. Smith EE (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the  
687 airways of cystic fibrosis patients. *Proc Natl Acad Sci* 103(22):8487–8492.
- 688 42. Andersen SB, Marvig RL, Molin S, Krogh Johansen H, Griffin AS (2015)  
689 Long-term social dynamics drive loss of function in pathogenic bacteria.  
690 *Proc Natl Acad Sci* 112(34):10756–10761.
- 691 43. Sommer LM, Molin S, Johansen HK, Marvig RL (2015) Convergent  
692 evolution and adaptation of *Pseudomonas aeruginosa* within patients with  
693 cystic fibrosis. *Nat Genet* 47(1):57–65.
- 694 44. Nguyen AT, et al. (2014) Adaptation of iron homeostasis pathways by a  
695 *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. *J*  
696 *Bacteriol* 196(12):2265–2276.
- 697 45. Folkesson A, et al. (2012) Adaptation of *Pseudomonas aeruginosa* to the  
698 cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol*  
699 10(12):841–851.
- 700 46. Zhang X-X, Rainey PB (2013) Exploring the sociobiology of pyoverdin-  
701 producing *Pseudomonas*. *Evolution* 67(11):3161–74.

- 702 47. Mellbye B, Schuster M (2014) Physiological framework for the regulation  
703 of quorum sensing-dependent public goods in *Pseudomonas aeruginosa*.  
704 *J Bacteriol* 196(6):1155–1164.
- 705 48. De Vos D, et al. (2001) Study of pyoverdine type and production by  
706 *Pseudomonas aeruginosa* isolated from cystic fibrosis patients:  
707 Prevalence of type II pyoverdine isolates and accumulation of pyoverdine-  
708 negative mutations. *Arch Microbiol* 175(5):384–388.
- 709 49. Visca P, Imperi F, Lamont IL (2007) Pyoverdine siderophores: from  
710 biogenesis to biosignificance. *Trends Microbiol* 15(1):22–30.
- 711 50. Cox CD, Adams P (1985) Siderophore activity of pyoverdin for  
712 *Pseudomonas aeruginosa*. *Infect Immun* 48(1):130–138.
- 713 51. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML (2002) Siderophore-  
714 mediated signaling regulates virulence factor production in *Pseudomonas*  
715 *aeruginosa*. *Proc Natl Acad Sci U S A* 99(10):7072–7077.
- 716 52. Griffin AS, West S a, Buckling A (2004) Cooperation and competition in  
717 pathogenic bacteria. *Nature* 430(August):1024–1027.
- 718 53. Dumas Z, Kümmerli R (2012) Cost of cooperation rules selection for  
719 cheats in bacterial metapopulations. *J Evol Biol* 25(3):473–484.
- 720 54. Dumas Z, Ross-Gillespie A, Kümmerli R (2013) Switching between  
721 apparently redundant iron-uptake mechanisms benefits bacteria in  
722 changeable environments. *Proc Biol Sci* 280(1764):20131055.
- 723 55. Popat R, Harrison F, McNally L, Williams P, Diggle SP (2016)  
724 Environmental modification via a quorum sensing molecule influences the  
725 social landscape of siderophore production. *bioRxiv*. doi:10.1101/053918.
- 726 56. Lee J, Zhang L (2014) The hierarchy quorum sensing network in  
727 *Pseudomonas aeruginosa*. *Protein Cell* 6(1):26–41.
- 728 57. Luria S, Delbrück M (1943) Mutations of Bacteria from Virus Sensitivity to  
729 Virus Resistance. *Genetics* 28(6):491–511.
- 730 58. Lee DH, Feist AM, Barrett CL, Palsson B (2011) Cumulative number of  
731 cell divisions as a meaningful timescale for adaptive laboratory evolution  
732 of escherichia coli. *PLoS One* 6(10):1–8.
- 733 59. Brown SP, West SA, Diggle SP, Griffin AS (2009) Social evolution in  
734 micro-organisms and a Trojan horse approach to medical intervention

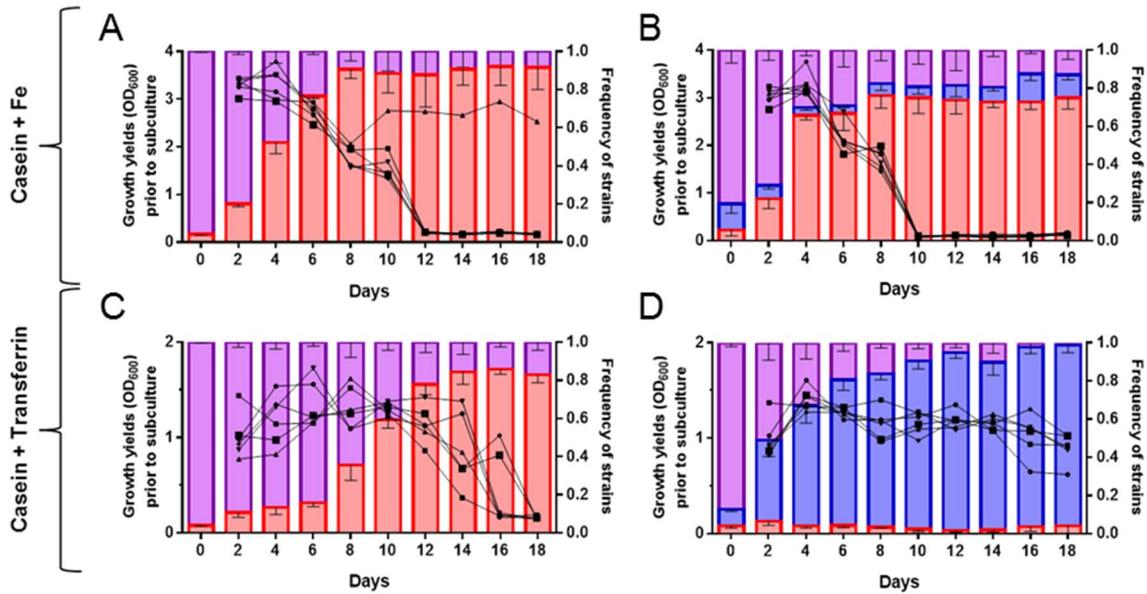
- 735 strategies. *Philos Trans R Soc Lond B Biol Sci* 364(1533):3157–3168.
- 736 60. Kümmerli R (2015) Cheat invasion causes bacterial trait loss in lung  
737 infections. *Proc Natl Acad Sci* 112(34):10577–10578.
- 738 61. Popat R, et al. (2012) Quorum-sensing and cheating in bacterial biofilms.  
739 *Proc Biol Sci* 279(1748):4765–4771.
- 740 62. Jiricny N, et al. (2010) Fitness correlates with the extent of cheating in a  
741 bacterium. *J Evol Biol* 23(4):738–747.
- 742 63. Ghoul M, West SA, Diggle SP, Griffin AS (2014) An experimental test of  
743 whether cheating is context dependent. *J Evol Biol* 27(3):551–556.



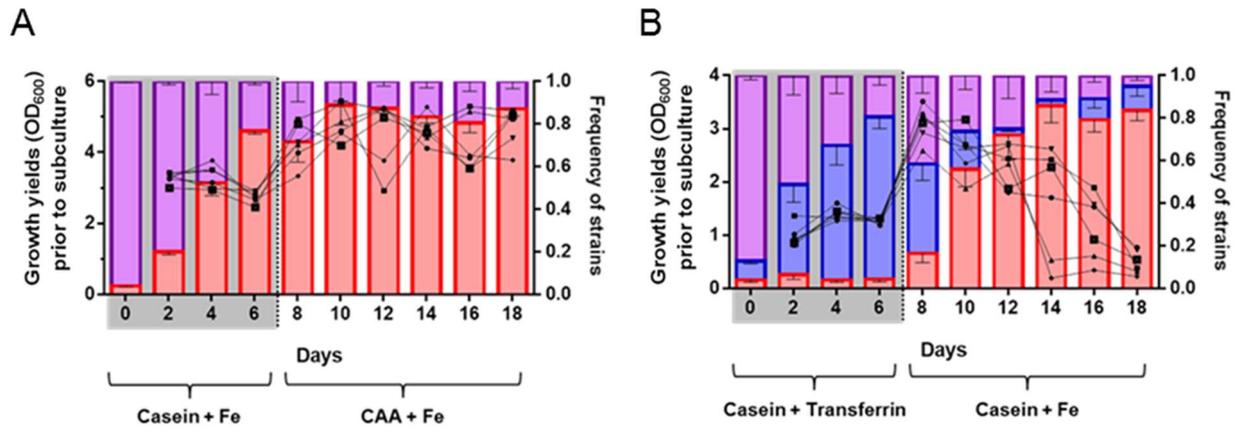
**Fig. 1.** Growth yields of *P. aeruginosa* monocultures in iron-depleted casein medium. WT (purple triangles), *pvdS* (blue squares) and *lasR* (red circles) mutant strains (horizontal lines show means of each group, Kruskal-Wallis test with Dunn's correction, WT-*pvdS* \*=P=0.011, WT-*lasR* \*\*\*\*=P<10<sup>-3</sup>, N=12).



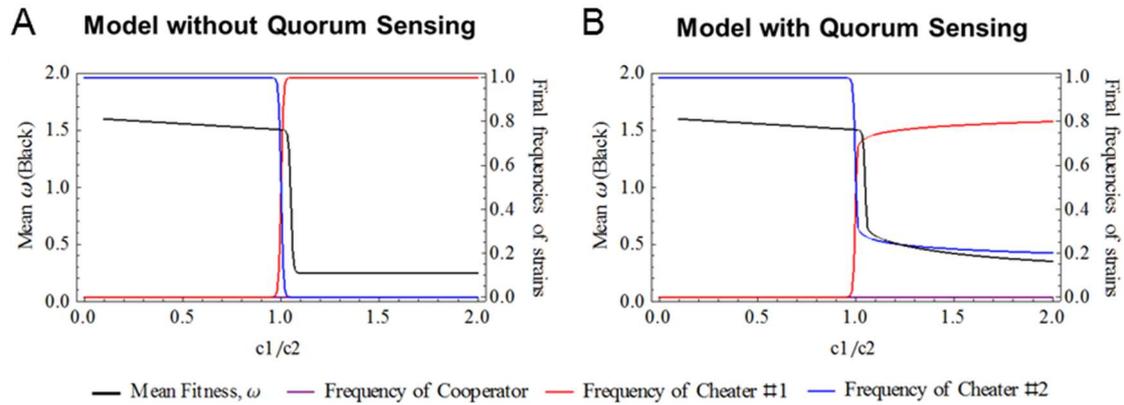
**Fig. 2.** Relative fitness of *lasR* or *pvdS* in iron-supplied or iron-depleted casein media in double or triple co-cultures. (A) Relative fitness of *lasR* (circles) in co-culture with WT, or with WT and *pvdS* in iron-supplied casein media (ns=not significant,  $P=0.1207$ ,  $N=20$ ). (B) Relative fitness of *pvdS* (squares) in co-culture with WT, or with WT and *lasR* in iron-supplied casein media (ns,  $P=0.1600$ ,  $N=12$ ). (C) and (D) same as in (A) and (B), respectively, but in iron-depleted casein media (\*\*\*\*= $P<10^{-3}$ ,  $N=15$  and ns,  $P=0.2581$ ,  $N=9$ , for (C) and (D), respectively). Relative fitness of *lasR* or *pvdS* mutants is calculated in respect to all the other strains in the population. Relative fitness  $> 1$  (above the dotted lines) indicate conditions where the frequency of the mutant increased in relation to the rest of the strains in the population during the competition. Initial ratios of the strains in each co-culture are 9:1 for WT:*lasR* and WT:*pvdS*, and 8:1:1 for WT:*pvdS*:*lasR*. Symbols indicate individual replicates and horizontal lines indicate the means of each group.



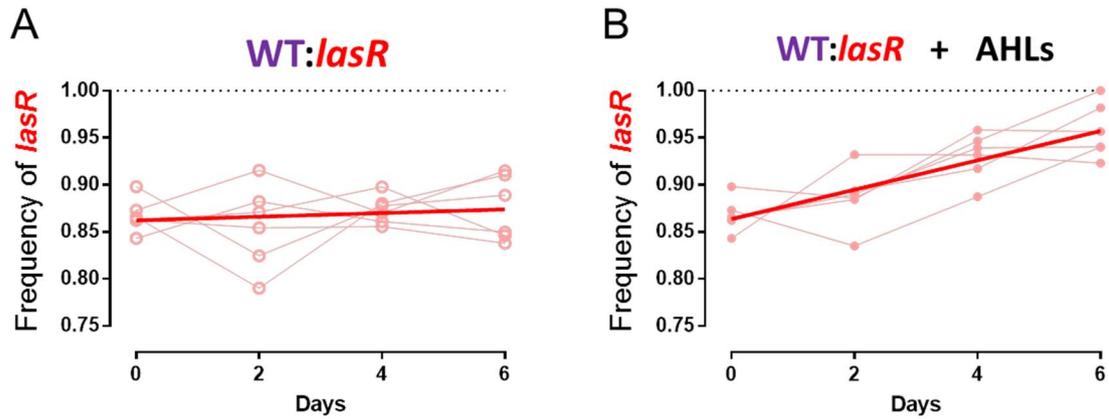
**Fig. 3.** Effects of abiotic and biotic factors on growth yields and strain composition of the population in long-term propagations. Left Y-axes show the OD<sub>600</sub> values prior to subculture; black symbols are the OD<sub>600</sub> values of 6 biological replicates tested for each condition (CFUs/ml are shown in Fig. S6). Right Y-axes show the frequencies of WT (purple), *lasR* (red), and *pvdS* (blue) mutants 48 hours after subculturing; data are shown as bars and represent the means of 6 biological replicates, error bars indicate SD. X-axes show the days of propagations to fresh media. (A) WT and *lasR* mutant co-cultures mixed at an initial frequency of 9:1 in iron-supplied casein media. (B) WT, *lasR* and *pvdS* mutants triple co-cultures mixed at initial an initial frequency of 8:1:1 in iron-supplied casein media. (C) and (D) same as in (A) and (B) but in iron-depleted casein media. Data from individual replicates are shown in Fig. S7 – 10.



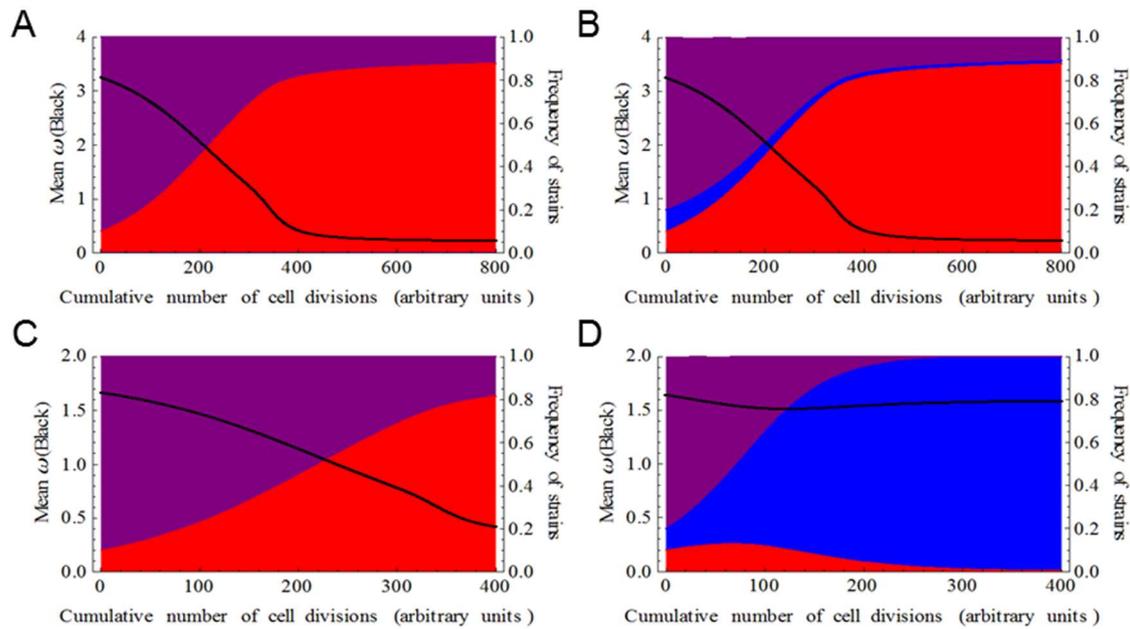
**Fig. 4.** Results of manipulations of abiotic conditions in long-term propagations. Aliquots of either the WT:*lasR* co-cultures propagated in iron-supplied casein media (Fig. 3A) or the WT:*lasR:pvdS* triple co-cultures propagated in iron-depleted casein media (Fig. 3D) for 6 days were transferred into either iron-supplied CAA medium or into iron-supplied casein medium, respectively. (A) Relief of complex carbon constraint by changing casein in iron-supplied casein medium to CAA, thus making it a medium with no constraints after the 6<sup>th</sup> day of the competitions (N=6, data from the first 6 days are from Fig. 3A). (B) Relief of low iron constraint by adding iron instead of iron depleting transferrin and changing iron-depleted casein medium into iron-supplied casein medium after the 6<sup>th</sup> day of the competitions (N=6, data from the first 6 days are from Fig. 3D). Legends as in Fig. 3.



**Fig. 5.** Mathematical model for the final frequencies of the three strains in relation to the ratio of  $c_1/c_2$ . In Right-Y axes, frequencies of cooperators of both cooperative traits (**purple**), cheater of the 1<sup>st</sup> cooperative trait (**red**), and cheater of the 2<sup>nd</sup> cooperative trait (**blue**) in relation to the ratio of  $c_1/c_2$  either without (**A**) or with the influence of quorum sensing (QS) regulation on the 1<sup>st</sup> cooperative trait (**B**). In Left-Y axes, the mean fitness,  $\bar{\omega}$ , is shown in **black**. The values given to the parameters of the simulations are:  $p_{\text{coop}}(0)=0.8$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $0.001 \leq c_1 < 0.199$ ,  $b_1=1.5$ ,  $c_2=0.1$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ , time (as arbitrary CCD)=1800. In (**B**) the 1<sup>st</sup> cooperative trait is regulated by QS with  $n=30$  and  $\text{th}=0.8$ .



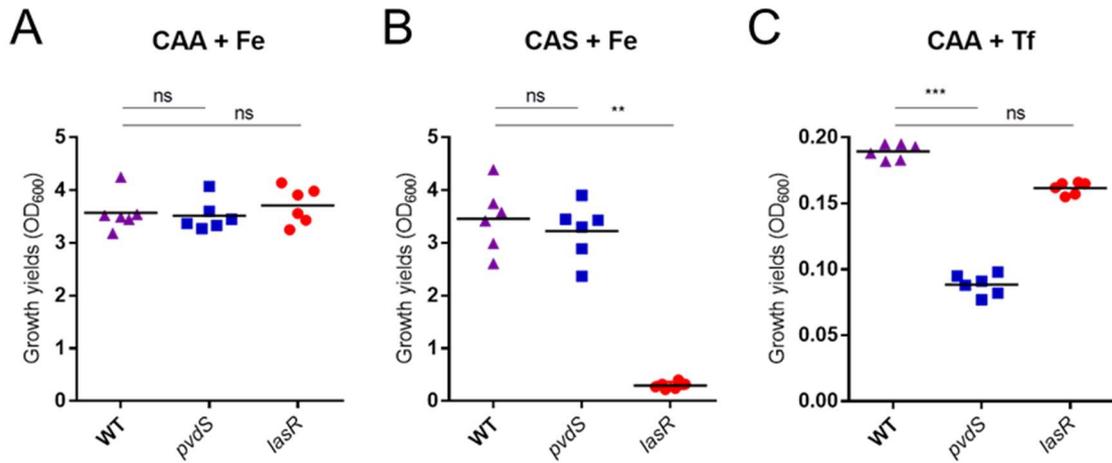
**Fig. 6.** Frequencies of *lasR* in propagations of WT:*lasR* co-cultures in iron-supplied casein media in the absence or presence of exogenously added AHLs (3OC<sub>12</sub>-HSL). Initial frequency of 80-90% of *lasR* were used. Cultures were propagated throughout 6 days by passing the fresh media each 48 hours. **(A)** Frequency changes of *lasR* in WT:*lasR* co-cultures (red). **(B)** is the same as (A) but with 5 $\mu$ M AHLs (3OC<sub>12</sub>-HSL) added to the media. Red lines indicate linear regressions of the data. Dotted lines represent 100% domination of *lasR*.



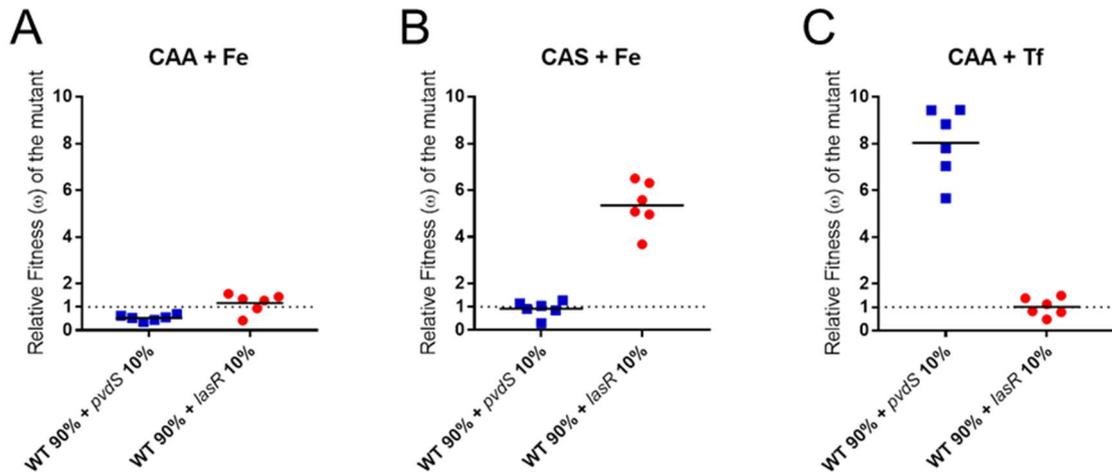
**Fig. 7.** Results of the mathematical model simulating the four scenarios in Fig. 3. Model includes quorum sensing regulation of the 1<sup>st</sup> cooperative trait ( $b_1$  and  $c_1$  are negatively regulated via a Hill equation as a function of the frequency of the mutant of this trait,  $p_{ch1}$ ). Left Y-axes show  $\bar{\omega}$ , the mean fitness of the entire population which is a proxy of OD<sub>600</sub> or CFUs/ml values prior to subculture (black lines). Right Y-axes show the frequencies of  $p_{coop}$  (e.g. WT, purple),  $p_{ch1}$  (e.g. *lasR*, red) and  $p_{ch2}$  (e.g. *pvdS*, blue). X-axes show the number of cell divisions as arbitrary units. The values that are given to the parameters of the simulations are: (A)  $p_{coop}(0)=0.9$ ,  $p_{ch1}(0)=0.1$ ,  $p_{ch2}(0)=0$ ,  $c_1=0.01$ ,  $b_1=3.4$ ,  $c_2=0$ ,  $b_2=0$ ,  $\omega_0=0.2$ ,  $th=0.8$ ,  $n=30$ ; (B)  $p_{coop}(0)=0.8$ ,  $p_{ch1}(0)=0.1$ ,  $p_{ch2}(0)=0.1$ ,  $c_1=0.01$ ,  $b_1=3.4$ ,  $c_2=0$ ,  $b_2=0$ ,  $\omega_0=0.2$ ,  $th=0.8$ ,  $n=30$ ; (C)  $p_{coop}(0)=0.9$ ,  $p_{ch1}(0)=0.1$ ,  $p_{ch2}(0)=0$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ ,  $th=0.8$ ,  $n=30$ ; (D)  $p_{coop}(0)=0.8$ ,  $p_{ch1}(0)=0.1$ ,  $p_{ch2}(0)=0.1$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ ,  $th=0.8$ ,  $n=30$ .

**Table 1:** Parameters for the mathematical model for the 3-way public goods game.

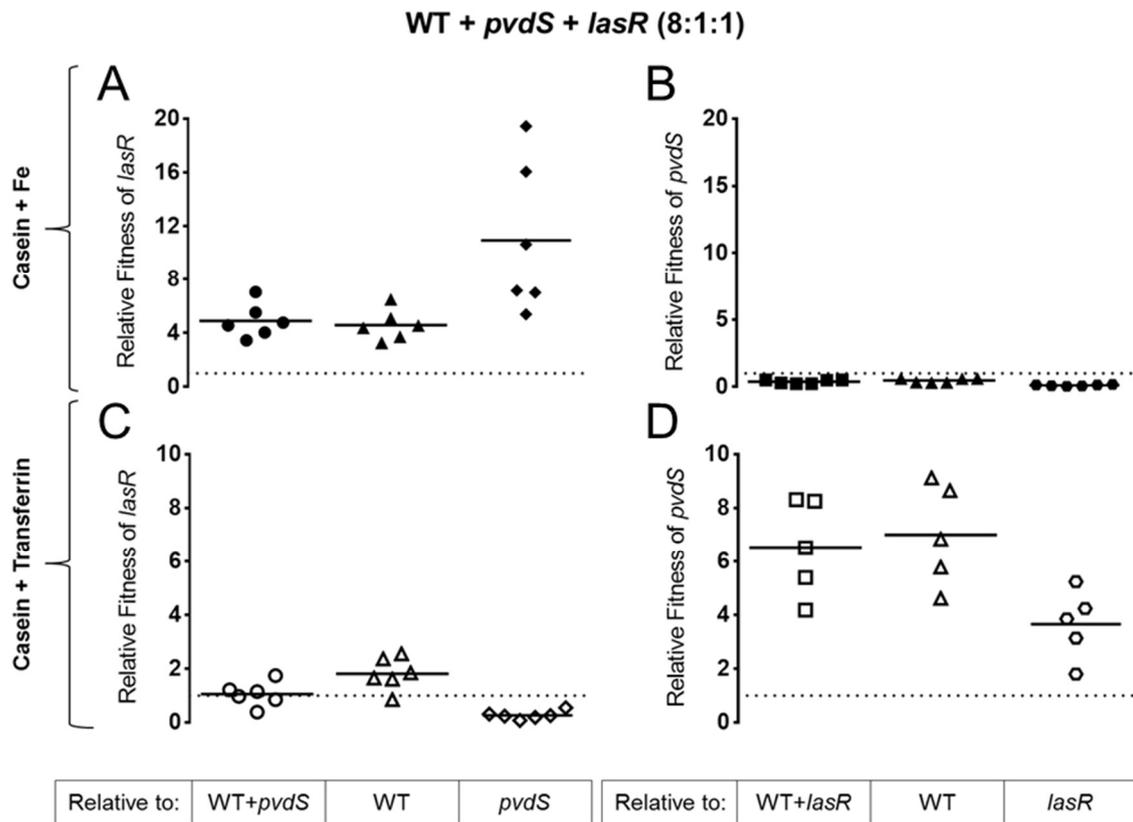
<b>Symbols</b>	<b>Descriptions</b>
$c_1$	Cost of the 1 <sup>st</sup> cooperative trait
$c_2$	Cost of the 2 <sup>nd</sup> cooperative trait
$b_1$	Benefit gained from the 1 <sup>st</sup> cooperative trait
$b_2$	Benefit gained from the 2 <sup>nd</sup> cooperative trait
$\omega_0$	Fitness without the additional fitness effects of the cooperative traits (basal fitness)
$\omega_{coop}$	Fitness of the cooperator of the both cooperative traits
$\omega_{ch1}$	Fitness of the cheater of the 1 <sup>st</sup> cooperative trait
$\omega_{ch2}$	Fitness of the cheater of the 2 <sup>nd</sup> cooperative trait
$\bar{\omega}$	Mean fitness of the entire population (A proxy for OD <sub>600</sub> or CFUs/ml)
$p_{coop}$	Frequency of the cooperator of the both cooperative traits in the entire population
$p_{ch1}$	Frequency of the cheater of the 1 <sup>st</sup> cooperative trait in the entire population
$p_{ch2}$	Frequency of the cheater of the 2 <sup>nd</sup> cooperative trait in the entire population
<b>Parameters considered only in the model with QS</b>	
$th$	Quorum threshold (as a function of the non-QS strain frequency)
$n$	Hill coefficient for the slope of the inhibition of the QS-regulated public good



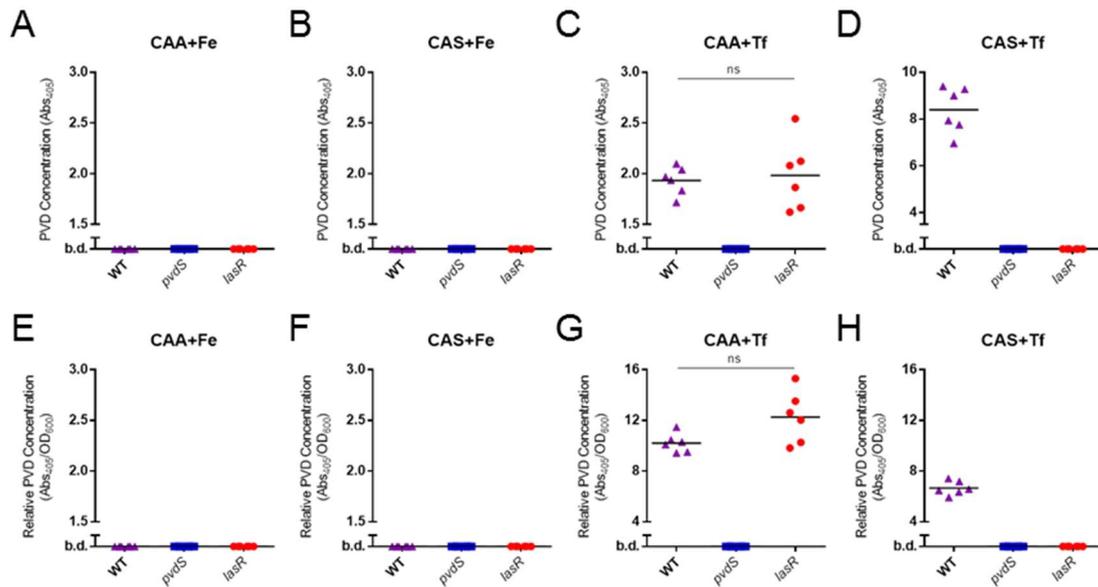
**Fig. S1:** Growth yields (OD<sub>600</sub>) of WT (purple triangles), *pvdS* (blue squares) and *lasR* (red circles) strains of *P. aeruginosa* monocultures after 48 hours of incubation in various media. **(A)** WT, *pvdS*, and *lasR* growth yields in iron-supplied casamino acids (CAA) medium (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* ns=not significant  $P>0.9999$ , WT-*lasR* ns=not significant  $P>0.9999$ , N=6). **(B)** WT, *pvdS*, and *lasR* growth yields in iron-supplied casein medium (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* ns=not significant  $P>0.9999$ , WT-*lasR* \*\*= $P=0.0034$ , N=6). **(C)** WT, *pvdS*, and *lasR* growth yields in iron-depleted CAA medium. (Kruskal-Wallis test with Dunn's correction, WT-*pvdS* \*\*\*= $P=0.0002$ , WT-*lasR* ns=not significant  $P=0.1027$ , N=6).



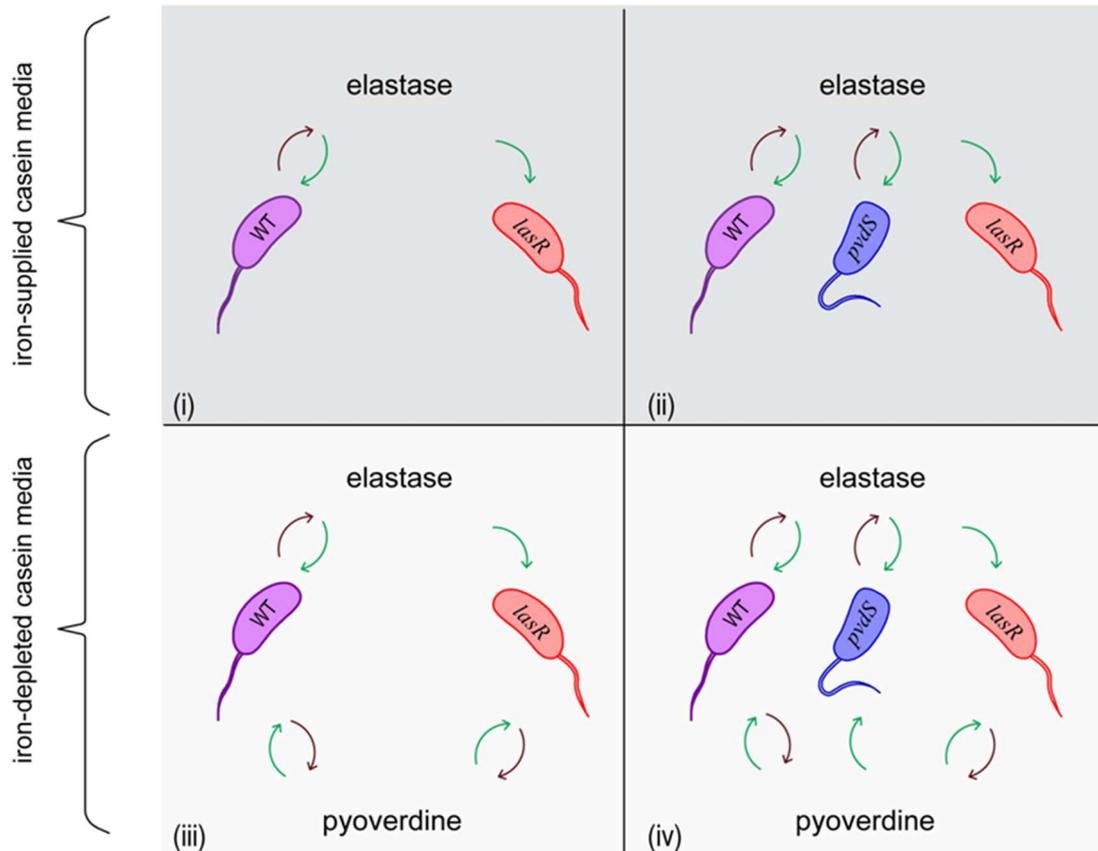
**Fig. S2:** Relative fitness of the mutants in co-cultures with WT with initial frequencies 9:1 WT:*pvdS* (blue squares) or 9:1 WT:*lasR* (red circles) after 48 hours of incubation in various media. **(A)** Relative fitness of *pvdS* and *lasR* in iron-supplied CAA medium (N=6). **(B)** Relative fitness of *pvdS* and *lasR* in iron-supplied casein medium (N=6). **(C)** Relative fitness of *pvdS* and *lasR* in iron-depleted CAA medium (N=6). Dotted lines indicate no change in relative fitness (relative fitness=1).



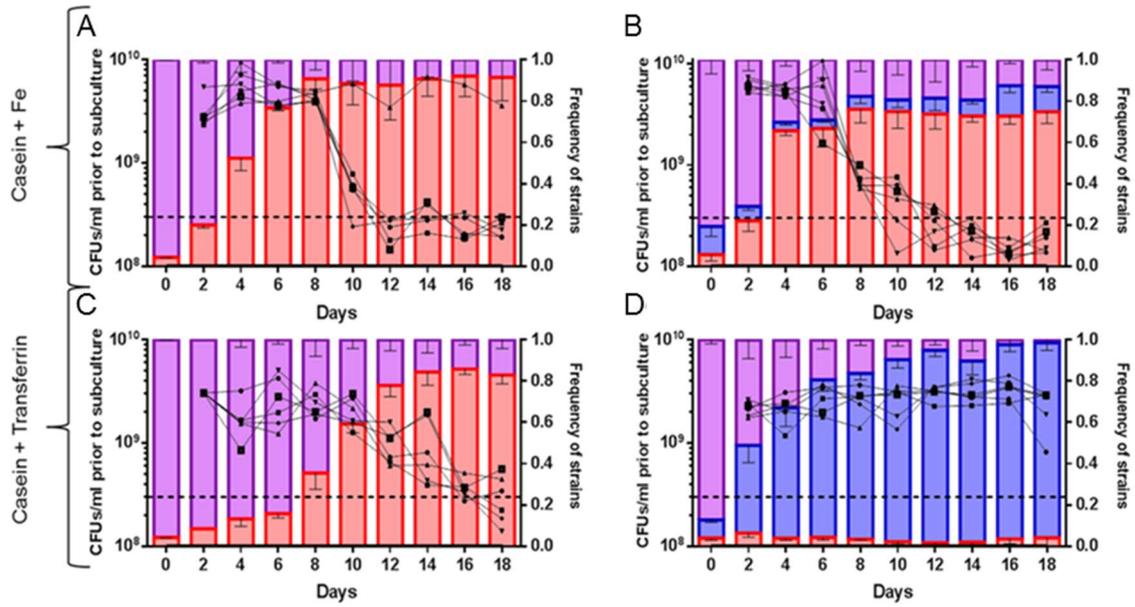
**Fig. S3.** Fitnesses of *lasR* and *pvdS* relative to the rest of the population, or to WT, or the other mutant in iron-supplied or iron-depleted casein media in WT:*pvdS*:*lasR* triple co-culture with the initial frequencies 8:1:1 (Data from Fig. 2). **(A)** Fitness of *lasR*, in iron-supplied casein media, relative to WT:*pvdS* (circles), WT (triangles) and *pvdS* (diamonds). **(B)** Fitness of *pvdS*, in iron-supplied casein media, relative to WT:*lasR* (squares), WT (filled) and *lasR* (hexagons). **(C)** and **(D)** same as in **(A)** and **(B)** (but with empty symbols), respectively, but in iron-depleted casein media. Symbols indicate individual replicates and horizontal lines indicate the means of each group. Dotted lines indicate no change in relative fitness (relative fitness=1).



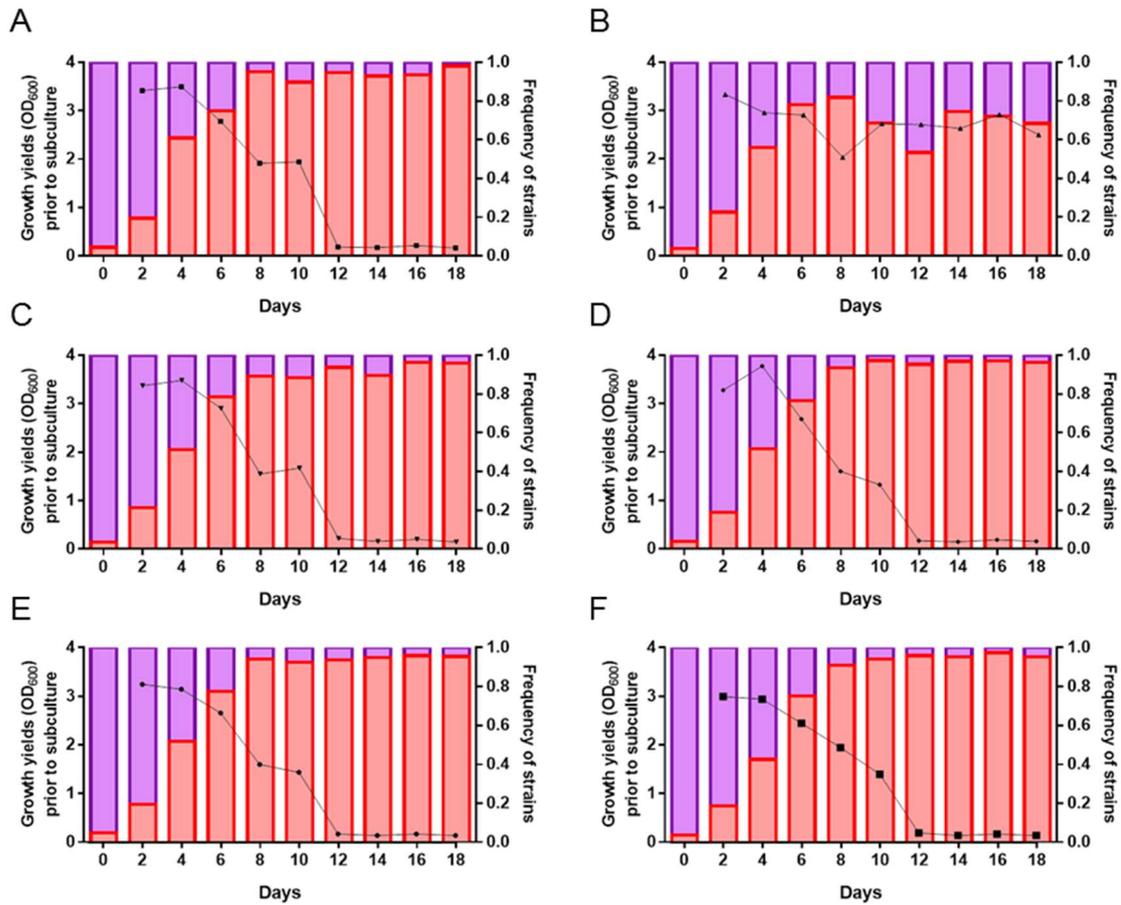
**Fig. S4.** Total (Abs<sub>405</sub>) and relative (Abs<sub>405</sub>/OD<sub>600</sub>) pyoverdine (PVD) concentrations of WT, *pvdS*, and *lasR* monocultures after 48 hours of incubation in various media. Total PVD concentrations (Abs<sub>405</sub>), **(A)** in iron-supplied CAA medium, **(B)** in iron-supplied casein medium, **(C)** in iron-depleted CAA medium, **(D)** in iron-depleted casein medium. Relative PVD concentrations (Abs<sub>405</sub>/OD<sub>600</sub>), **(E)** in iron-supplied CAA medium, **(F)** in iron-supplied casein medium, **(G)** in iron-depleted CAA medium, **(H)** in iron-depleted casein medium. (Comparisons are done via Mann-Whitney test; ns=not significant, P>0.05; for all experiments N=6; b.d.: below detection). **Methodology.** PVD concentration measurements are done after 48 hours of growth in 37°C shaker by centrifuging the cells at 14000 r.p.m. for 4 minutes (Eppendorf Centrifuge 5418) and collecting the supernatant, measuring their absorbance at 405nm (Abs<sub>405</sub>) in optical cuvettes as 1:10 dilutions with PBS solutions in a Thermo Spectronic Helios δ spectrophotometer.



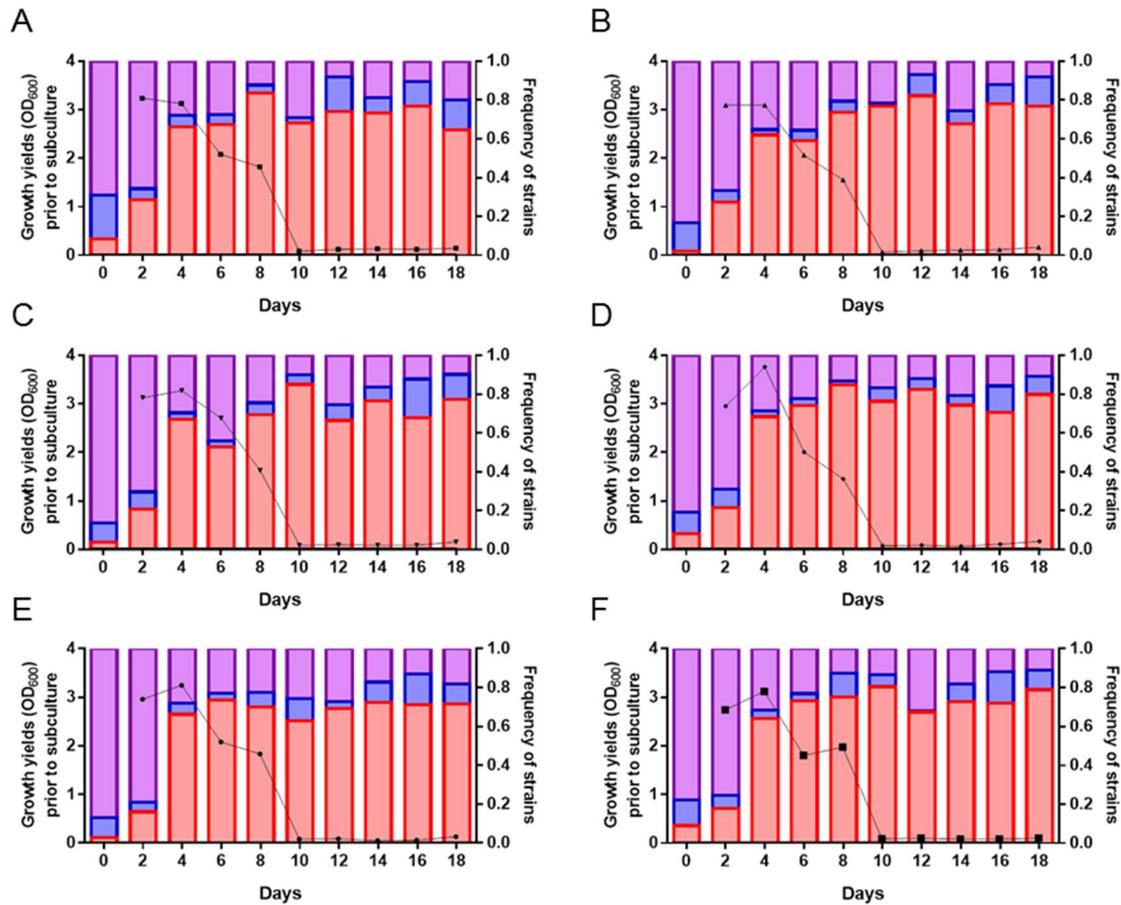
**Fig. S5.** Four scenarios representing the environmental constraints and population compositions tested. **i)** double co-culture in iron-supplied casein medium (WT and one mutant, under one environmental constraint); **ii)** triple co-culture in iron-supplied casein medium (WT and two mutants, under one environmental constraint); **iii)** double co-culture in iron-depleted casein medium (WT and one mutant, under two environmental constraints); **iv)** triple co-culture in iron-depleted casein medium (WT and two mutants, under two environmental constraints). Dark-red arrows indicate a paid cost for the production of elastase and/or pyoverdine, and the green arrows indicate a benefit from these behaviors. Dark-grey backgrounds indicate optimal iron concentrations; light-grey backgrounds indicate depleted iron concentrations in the media.



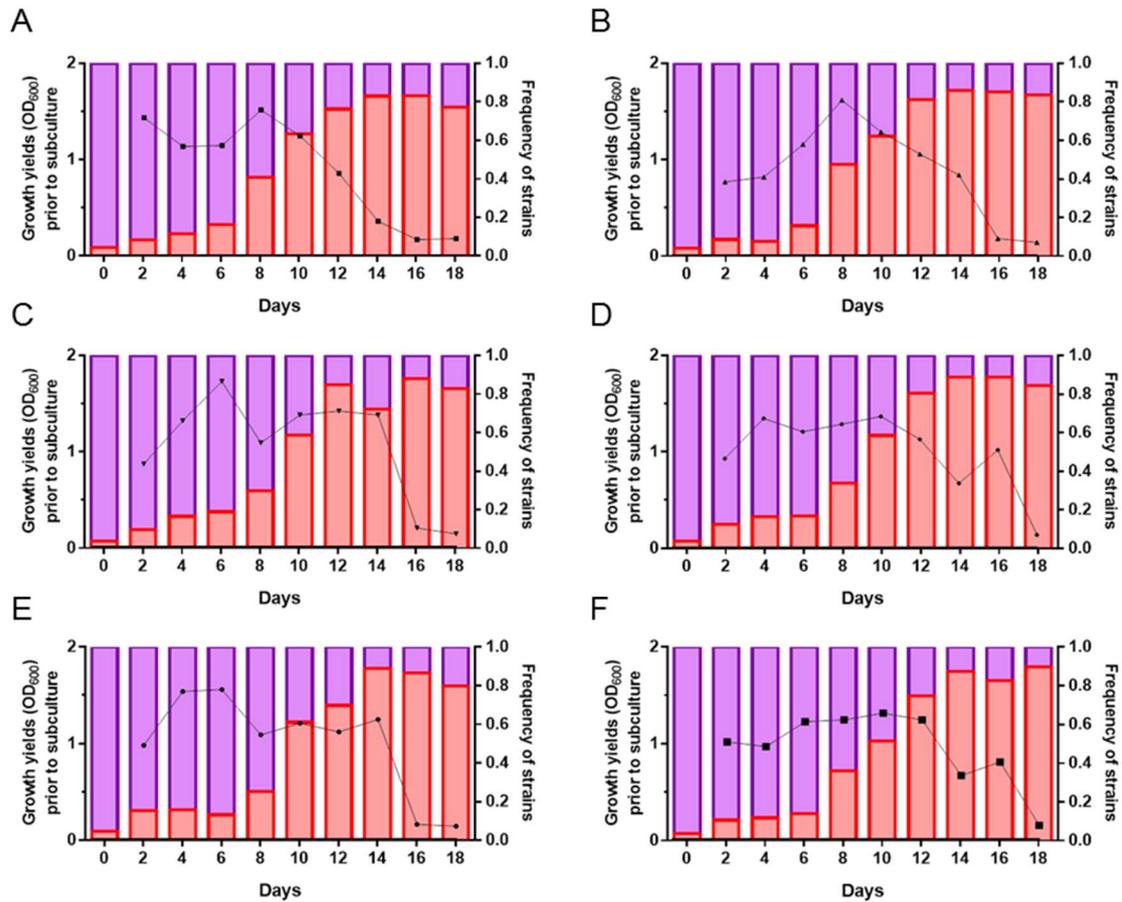
**Fig. S6.** Data from the experiments shown in Fig. 3 with the growth yields prior to subculture, shown as CFUs/ml. Dash lines indicate the approximate monoculture growth yields of *lasR* monoculture ( $3 \times 10^8$  CFUs/ml).



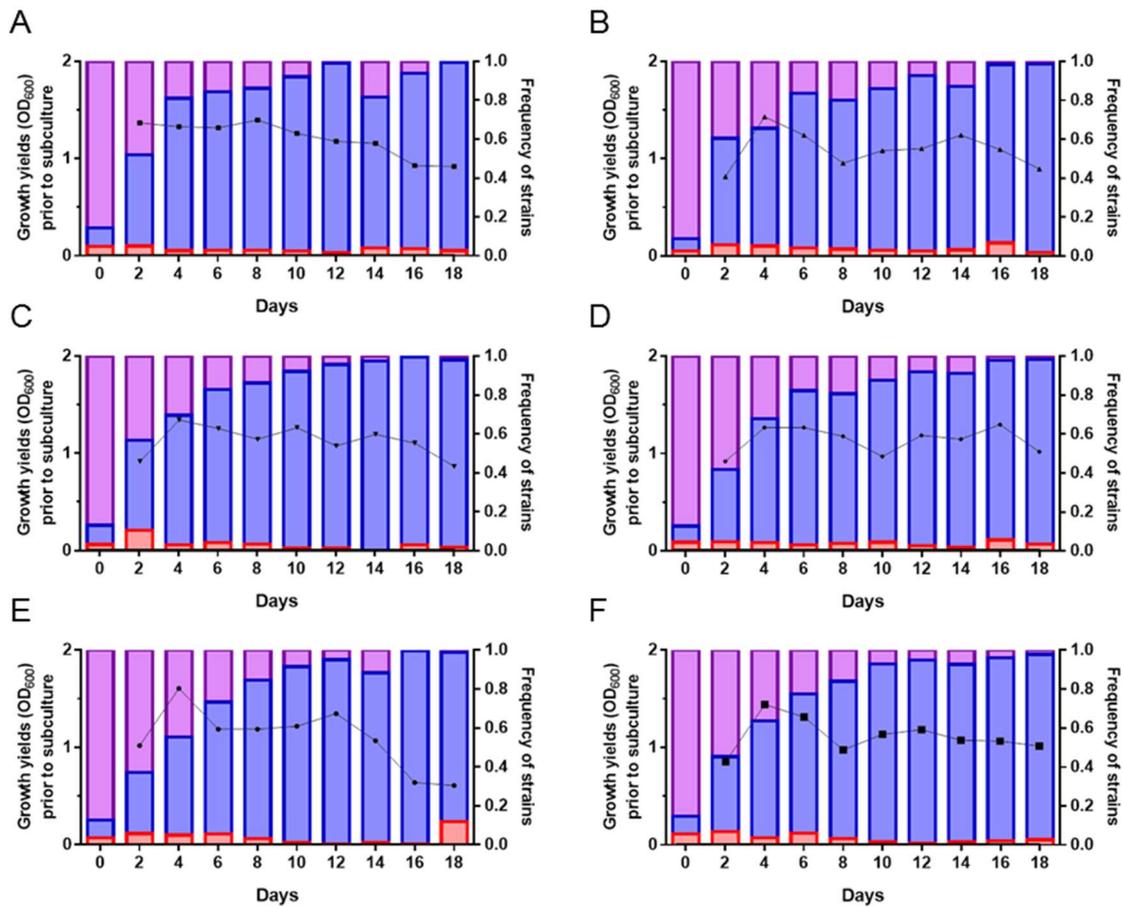
**Fig. S7.** Individual biological replicates from the Fig. 3A. WT:*lasR* populations, which are co-cultured with initial frequencies of 9:1, in iron-supplied casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD<sub>600</sub>), prior to subculture; data shown as black lines are the growth yields (OD<sub>600</sub>) of the cultures measured at the late stationary phase (48 hour after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple) and *lasR* (red).



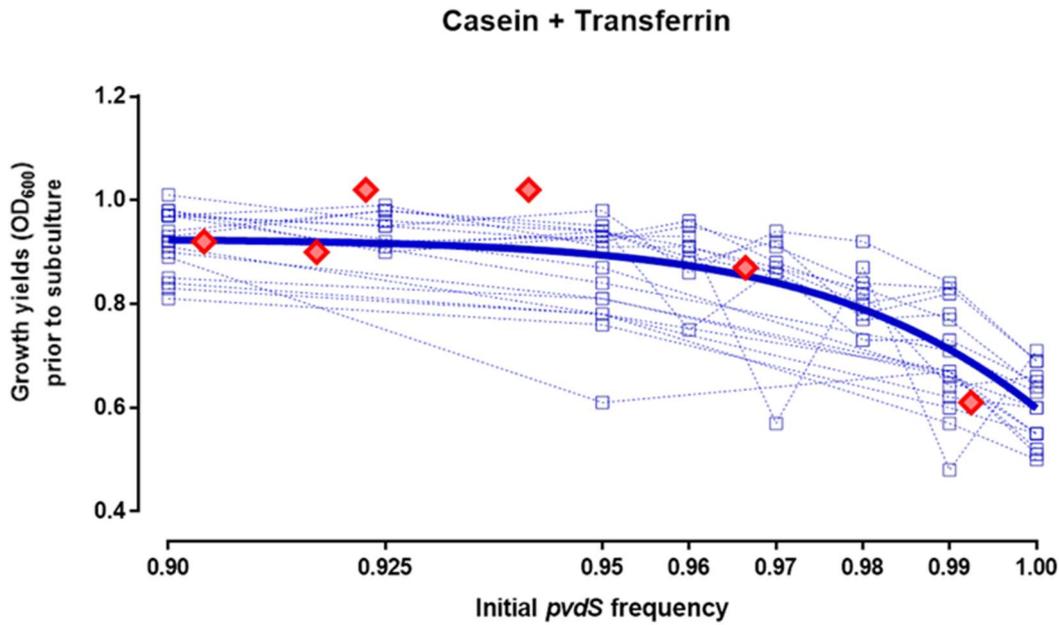
**Fig. S8.** Individual biological replicates from the Fig. 3B. WT:*lasR*:*pvdS* populations, which are co-cultured with initial frequencies of 8:1:1, in iron-supplied casein media.. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD<sub>600</sub>) prior to subculture; data shown as black lines are the growth yields (OD<sub>600</sub>) of the cultures measured at the late stationary phase (48 hour after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple), *pvdS* (blue), and *lasR* (red).



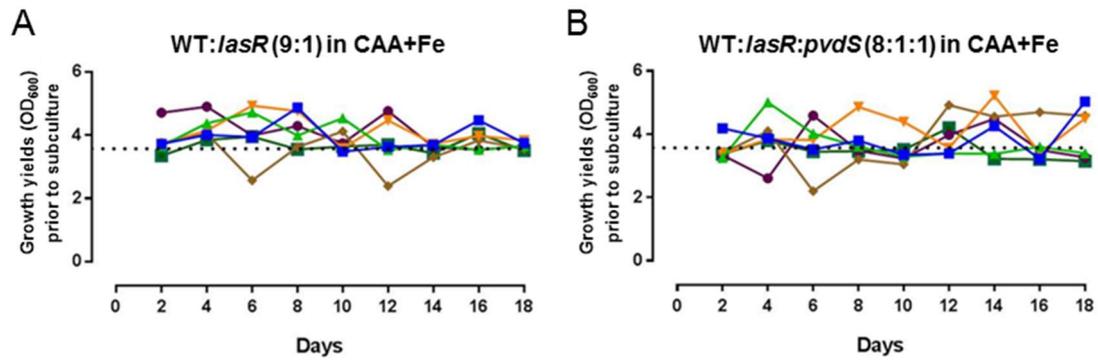
**Fig. S9:** Individual biological replicate from the Fig. 3C. WT:*lasR* populations, which are co-cultured with initial frequencies of 9:1, in iron-depleted casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD<sub>600</sub>) prior to subculture; data shown as black lines are the growth yields (OD<sub>600</sub>) of the cultures measured at the late stationary phase (48 hour after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple) and *lasR* (red).



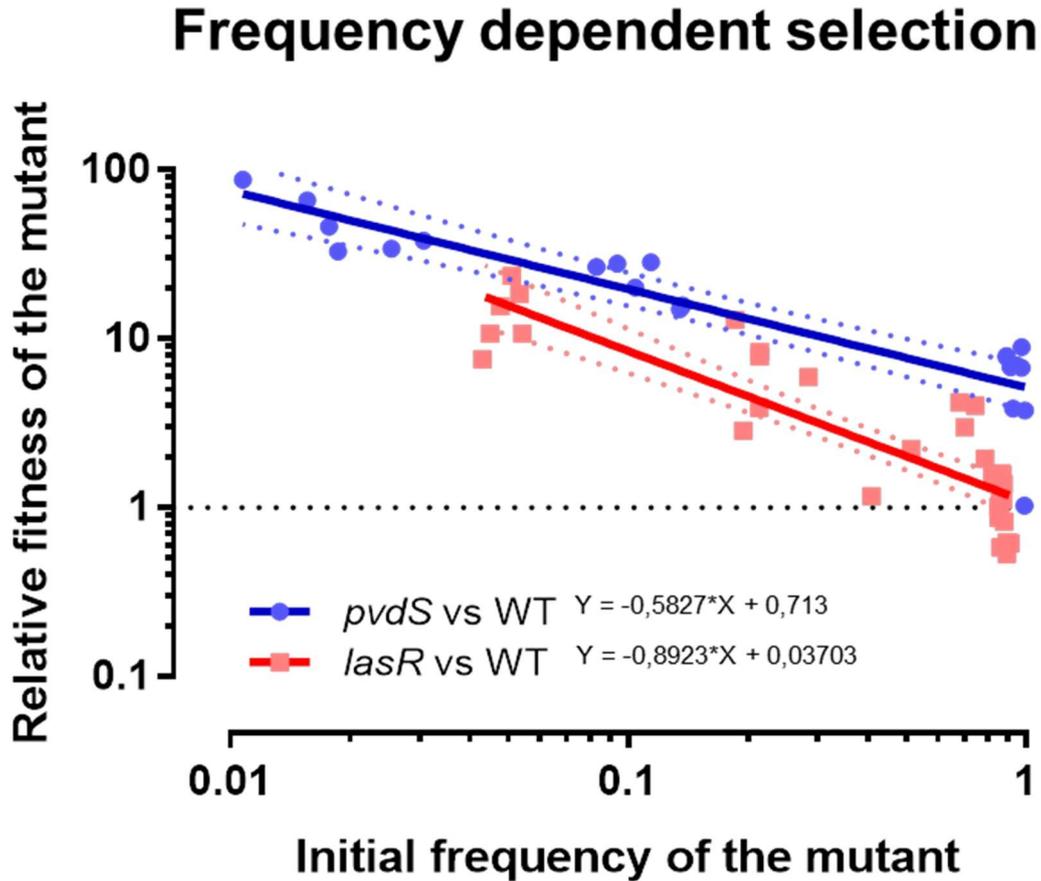
**Fig. S10.** Individual biological replicates from the Fig. 3D. WT:*lasR*:*pvdS* populations, which are co-cultured with initial frequencies of 8:1:1, in iron-depleted casein media. 'X' axes show the days of propagations to fresh media. Left 'Y' axes show the growth yields (OD<sub>600</sub>) prior to subculture; data shown as black lines are the growth yields (OD<sub>600</sub>) of the cultures measured at the late stationary phase (48 hour after the inoculation) values. Right 'Y' axes show the frequencies of WT (purple), *pvdS* (blue), and *lasR* (red).



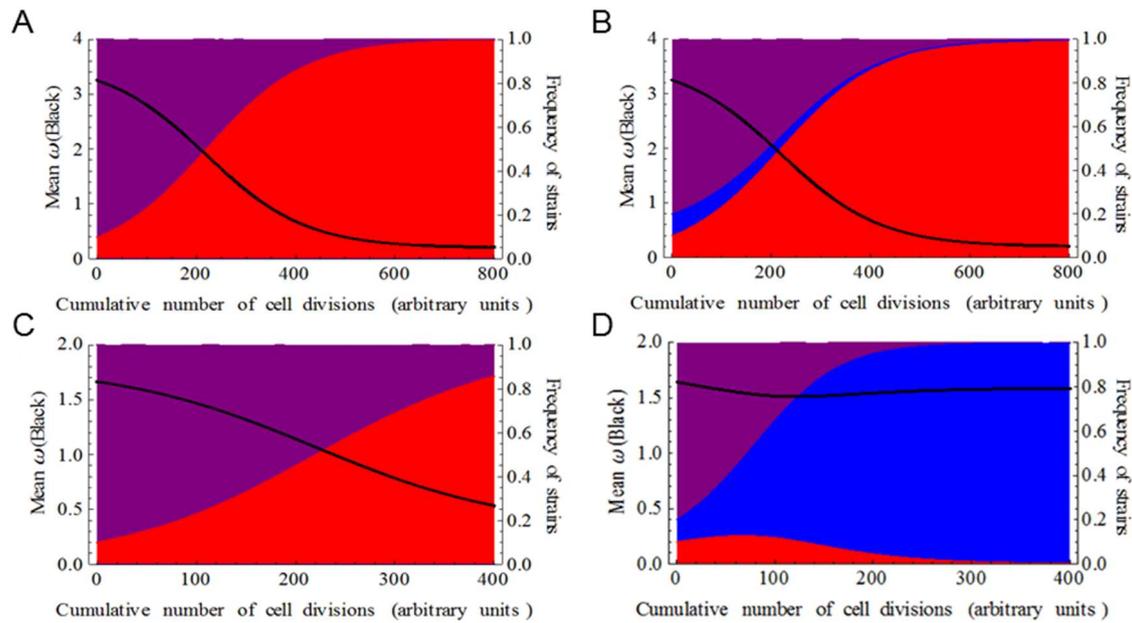
**Fig. S11.** Effect of the initial frequencies of *pvdS* mutant in the co-cultures with WT, on the overall growth yields of the population. Each blue square represents one short term competition (48 hours) in iron-depleted casein media. Initial frequencies of *pvdS* are shown in the 'X' axis (curve indicates the log regression of these short term competitions). Red diamonds show the OD<sub>600</sub> measurements and matching inoculum frequencies of *pvdS* mutant from different co-cultures of the 18<sup>th</sup> day of the experiment shown in Fig. 3D (OD<sub>600</sub> of the cultures with 3% WT and 97% *pvdS* vs. 100% WT, P=0.1316; and OD<sub>600</sub> of the cultures with 2% WT and 98% *pvdS* vs 100% WT, P<0.05).



**Fig. S12.** Propagations of *P. aeruginosa* cultures in a medium with no constraints. **(A)** WT and *lasR* co-culture initially mixed (9:1) in iron-supplied CAA media. **(B)** WT, *pvdS* and *lasR* co-culture initially mixed (8:1:1) in iron-supplied CAA media. 'X' axes show the days of propagations to fresh media. 'Y' Axes show the growth yields as OD<sub>600</sub> prior to subculture, each colored line indicates one propagated culture (N=6), dash lines indicate the monoculture WT growth yields in the same medium (mean=3.57,  $\pm$ SD=0.357, N=6) as shown in Fig. S1A.



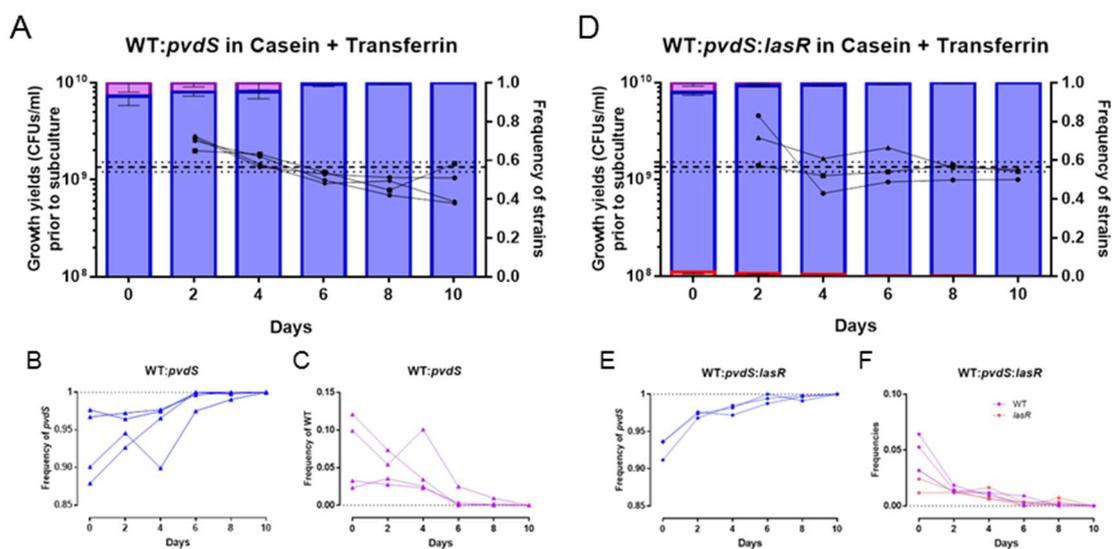
**Fig. S13.** Frequency-dependent selection for *pvdS* (blue circles) and *lasR* (red squares) when in co-culture with WT in iron-depleted or iron-supplied casein media for *pvdS* or *lasR*, respectively. 'X' axis shows the frequencies of *pvdS* or *lasR* in the beginning of the competition with WT. 'Y' axis shows the relative fitness values of *pvdS* or *lasR* over WT after 48h of incubation. Lines indicate linear regressions; slopes of the lines are shown on the figure (Comparison of the lines:  $F=8,525$ ,  $DFn=1$ ,  $DFd=52$ ,  $**=P=0,0052$ ; the lines are significantly different). Red and blue dots indicate the 95% confidence intervals of the corresponding lines. The gray dotted line indicates no change in relative fitness (no cheating, relative fitness=1).



**Fig. S14.** Four scenarios in Fig. 3 A – D simulated using the mathematical model for the 3-way public good game without QS regulation. Left Y-axes show  $\bar{\omega}$ , the mean fitness of the entire population, which is a proxy of OD<sub>600</sub> or CFUs/ml values prior to subculture (black lines). Right Y-axes show the frequencies of  $p_{\text{coop}}$  (e.g. WT, purple),  $p_{\text{ch1}}$  (e.g. *lasR*, red) and  $p_{\text{ch2}}$  (e.g. *pvdS*, blue). X-axes show the cumulative number of cell divisions as arbitrary units. The values that are given to the parameters of the simulations are: **(A)**  $p_{\text{coop}}(0)=0.9$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0$ ,  $c_1=0.01$ ,  $b_1=3.4$ ,  $c_2=0$ ,  $b_2=0$ ,  $\omega_0=0.2$ ; **(B)**  $p_{\text{coop}}(0)=0.8$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $c_1=0.01$ ,  $b_1=3.4$ ,  $c_2=0$ ,  $b_2=0$ ,  $\omega_0=0.2$ ; **(C)**  $p_{\text{coop}}(0)=0.9$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ ; **(D)**  $p_{\text{coop}}(0)=0.8$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ . Note that the values of parameters used in these simulations are chosen to reflect approximately the relation between the values observed in Fig. 1, Fig. S1, Fig. 2, and Fig. 3.

## Supplementary text supporting Fig. S15:

***pvdS* mutant can reach fixation.** Our mathematical model predicts that mutants which are winning in a competition (*lasR* or *pvdS*) would reach fixation in our propagation experiments. We experimentally tested whether this would happen if the propagation were prolonged in competitions with initial mutant frequencies similar to those at the 18<sup>th</sup> day of the competitions in Fig. 3D. The results in Fig. S15 below show that when the competitions are initiated with *pvdS* frequencies similar to those at day 18 in Fig. 3D, *pvdS* always reaches to fixation when co-cultured either with WT (Fig. S15 A, B, and C), or with WT and *lasR* mutant (Fig. S15 D, E, and F).



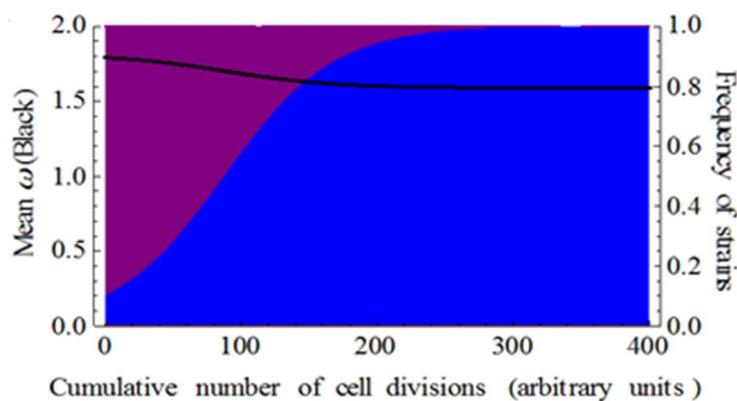
**Fig. S15.** Propagations of WT:*pvdS* ((A), (B), and (C)) and WT:*lasR*:*pvdS* cultures ((D), (E) and (F)) in iron-depleted casein media throughout 10 days by passing the cultures to fresh media with a 1/1000 dilution after each 48 hours of growth ('X' axes show the days of propagation and the initial frequencies are shown at day 0). (A) Frequency changes of WT (purple) and *pvdS* (blue) in WT:*pvdS* co-cultures shown as stacked bars (right 'Y' axes) as the growth yields (CFUs/ml) of 4 biological replicates are shown as black lines (left 'Y' axes). (B) A detailed presentation of the frequency changes of *pvdS* in WT:*pvdS* co-cultures (blue). (C) A detailed presentation of the frequency changes of WT in WT:*pvdS* co-cultures (purple). (D) Frequency changes of WT (purple), *pvdS* (blue), and *lasR* (red) in WT:*pvdS*:*lasR* co-cultures shown as stacked bars (right 'Y' axes) as the growth yields (CFUs/ml) of 3 biological replicates are shown as black lines (left 'Y' axes). (E) A detailed presentation of the frequency changes of *pvdS* in WT:*pvdS* co-cultures (blue). (F) A detailed presentation of the frequency

changes of WT (purple), and *lasR* (red), in WT:*pvdS*:*lasR* co-cultures. Dash lines indicate the mean monoculture *pvdS* growth yields in the same media and the dotted lines indicate SD in (A) and (D) (mean:  $1.35 \times 10^9$  CFUs/ml;  $\pm$ SD =  $1.12 \times 10^0$  CFUs/ml). Dotted lines indicate full fixation of *pvdS* to 100% of the population in (B), (C), (E) and (F). For (A), (B), and (C) N=4; and for (D), (E), and (F) N=3.

## Supplementary text supporting Fig. S16 – 19:

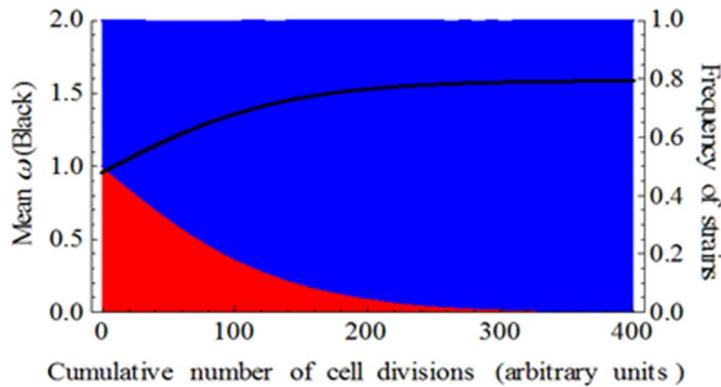
**Simulations of other possible scenarios.** We also simulated alternative scenarios in an environment where both traits are needed **without QS regulation** or **with QS regulation** (only the 1<sup>st</sup> cooperative trait is regulated by QS):

- (A) When only the cooperator of both cooperative traits (e.g. WT) and the cheater of the 2<sup>nd</sup> cooperative trait (which is not regulated by quorum sensing, e.g. *pvdS*) are in competition, the cheater wins and reaches to fixation as in the triple co-culture scenario, regardless of QS regulation of the 1<sup>st</sup> cooperative trait (**Fig. S16**), while the mean fitness becomes:  $\bar{\omega} = \omega_0 + b_1 - c_1$ ;



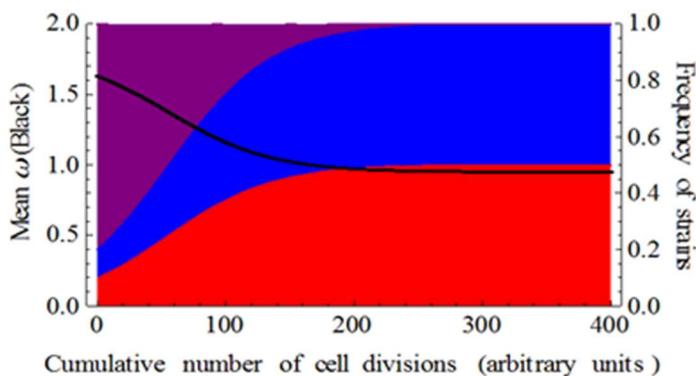
**Fig. S16.** Simulation with two strains, full cooperator (WT) + cheater for the 2<sup>nd</sup> cooperative trait, under conditions where both public goods are produced and  $c_2 > c_1$ . Left Y-axis show  $\bar{\omega}$ , the mean fitness of the entire population which is a proxy of OD<sub>600</sub> or CFUs/ml values prior to subculture (black lines). Right Y-axis show the frequencies of  $p_{\text{coop}}$  (purple), and  $p_{\text{ch2}}$  (blue). X-axis shows the cumulative number of cell divisions as arbitrary units. The values given to the parameters of the simulations were:  $p_{\text{coop}}(0)=0.9$ ,  $p_{\text{ch1}}(0)=0$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ . The results were the same regardless if the 1<sup>st</sup> was considered to be regulated by QS ( $n=30$ ,  $th=0.8$ ) or not ( $n=0$ ,  $th=0$ ).

- (B) When only **two cheaters are in 1:1 competition**, the cheater that saves the greater cost (here, the cheater of the 2<sup>nd</sup> cooperative trait since  $c_2 > c_1$ ) wins the competition and reaches to fixation regardless of QS regulation of the 1<sup>st</sup> cooperative trait (**Fig. S17**), while the mean fitness becomes:  $\bar{\omega} = \omega_0 + b_1 - c_1$ ;



**Fig. S17.** Simulation with the two cheaters competing with each other, under conditions where both public goods are produced and  $c_2 > c_1$ . Axes as in Fig. S16. Frequencies of  $p_{coop}$  (purple),  $p_{ch1}$  (red), and  $p_{ch2}$  (blue) are shown. The values given to the parameters of the simulations were:  $p_{coop}(0)=0$ ,  $p_{ch1}(0)=0.5$ ,  $p_{ch2}(0)=0.5$ ,  $c_1=0.01$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ . The results were the same regardless if QS regulation for the 1<sup>st</sup> cooperative trait was considered ( $n=30$ ,  $th=0.8$ ) or not ( $n=0$ ,  $th=0$ ).

**(C)** When the cooperator of the both cooperative traits (e.g. WT) is competing with two mutants under conditions where the costs of both traits are equal ( $c_1=c_2$ ), both cheaters increase in frequency until both of them reach 50% of the population (**Fig. S18**), similarly with or without QS regulation of the 1<sup>st</sup> cooperative trait, while the mean fitness becomes:  $\bar{\omega} = \omega_0 + \frac{1}{2} (b_1 + b_2 - c_1 - c_2)$ .



**Fig. S18.** Simulation for a 3-way competition with the cooperator of the both cooperative traits competing with two cheaters, under conditions where both public goods are produced and  $c_2=c_1$ . Axes as in Fig. S16. Frequencies of  $p_{coop}$  (purple),  $p_{ch1}$  (red), and  $p_{ch2}$  (blue) are shown. The values given to the parameters

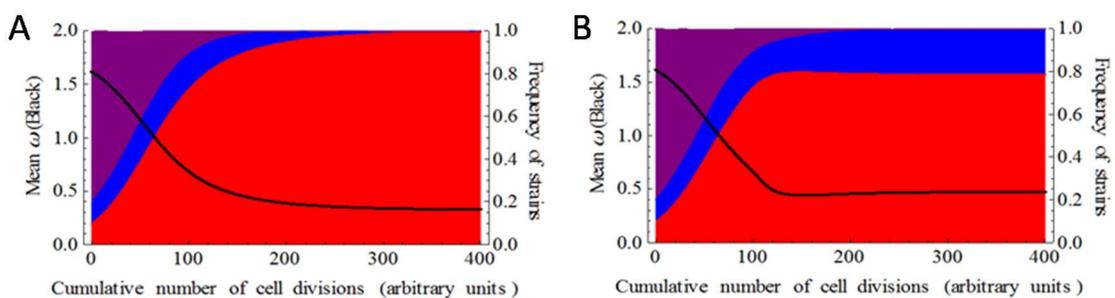
of the simulations were:  $p_{\text{coop}}(0)=0.8$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $c_1=0.025$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ . The results were the same regardless if QS regulation for the 1<sup>st</sup> cooperative trait was considered ( $n=30$ ,  $th=0.8$ ) or not ( $n=0$ ,  $th=0$ ).

**(D)** When the cooperator of the both cooperative traits (e.g. WT) is competing with two mutants under conditions where the  $c_1 > c_2$ , then the more drastic tragedy inducing cheater becomes the winner of the 3-way competition. In this case, when the 1<sup>st</sup> cooperative is not regulated by QS, the cheater of the 1<sup>st</sup> cooperative trait wins the 3-way competition and causes a drastic collapse, with the mean fitness:  $\bar{\omega} = \omega_0 + b_2 - c_2$  (**Fig. S19A**).

However, when the 1<sup>st</sup> cooperative trait is regulated by QS, the cheater of the 1<sup>st</sup> cooperative, while it still wins the competition, it can only increase in frequency until the QS threshold ( $th=0.8$ ) and thus, cannot reach to fixation (**Fig. S19B**); and the mean fitness becomes:

$$\omega_0 + (b_1 - c_1) (0.4) + (b_2 - c_2) (0.8)$$

As a result, the the cooperator of the both cooperative traits (e.g. WT) persists in the population. Therefore, presumably the population has a greater chance to recover, if the environmental conditions change. In conclusion, the QS regulation becomes relevant only under conditions where the mutant for the QS-regulated trait (here the cheater of the 1<sup>st</sup> cooperative trait) is not completely outcompeted.



**Fig. S19.** Simulation for a 3-way competition with the cooperator of the both cooperative traits competing with two cheaters, under conditions where both public goods are produced and  $c_1 > c_2$ . Axes as in Fig. S16. Frequencies of  $p_{\text{coop}}$  (purple),  $p_{\text{ch1}}$  (red) and  $p_{\text{ch2}}$  (blue) are shown. The values given to the parameters of the simulations were: **(A)**  $p_{\text{coop}}(0)=0.8$ ,  $p_{\text{ch1}}(0)=0.1$ ,  $p_{\text{ch2}}(0)=0.1$ ,  $c_1=0.04$ ,  $b_1=1.5$ ,  $c_2=0.025$ ,  $b_2=0.25$ ,  $\omega_0=0.1$ ,  $n = 0$ ,  $th = 0$ ; **(B)** same as in (A) except  $n=30$  and  $th=0.8$ , as QS regulation was considered for the 1<sup>st</sup> cooperative trait.