

1 **Cheating on cheaters dramatically affects social interactions in**
2 ***Pseudomonas aeruginosa***

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9 Running Head: Cheating on cheaters prevents drastic population collapse

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13 **Abstract**

14 Bacterial cooperation can be disrupted by non-producers, which can profit from
15 public goods without paying their production cost. A cheater can increase in
16 frequency, exhausting the public good and causing a population collapse. Here
17 we investigate how interactions among two cheaters for distinct social traits
18 influence the short and long-term dynamics of polymorphic populations. Using
19 as a model *Pseudomonas aeruginosa* and its extensively studied social traits,
20 production of the siderophore pyoverdine and the quorum sensing regulated
21 elastase, we analyzed the social dynamics of polymorphic populations under
22 conditions where the two traits are required for optimal growth. We show that
23 cheaters for either trait compete with both the wild type and each other, and that
24 mutants for pyoverdine production can prevent a drastic population collapse
25 caused by quorum sensing cheaters. A simple mathematical model suggests
26 that the observed social dynamics are determined by the ratio of the costs of
27 each social trait, such that the mutant which avoids paying the highest cost
28 dominates the population. Finally, we demonstrate how quorum sensing
29 regulation can avoid the full loss of cooperation.

30

31 Introduction

32 Bacteria are unicellular organisms, but can engage in diverse group
33 behaviors, including biofilm formation, swarming motility, and production of
34 extracellular proteases or iron-chelating siderophores [1–4]. The production of
35 compounds that can benefit both producers and non-producers (public goods)
36 can be considered as one of these cooperative behaviors. Cooperation is
37 frequently under the threat of exploitation by cheaters: individuals that benefit
38 from the cooperative action but contribute little or nothing to the production of
39 the public goods. This situation, where both cooperators and cheaters can
40 access a resource produced by the formers, is referred to as public goods
41 dilemma [5,6]. If cheaters emerge, by mutation or migration, they can increase
42 in frequency and cause loss of cooperation. As they rise to dominance, the
43 public goods get exhausted and a population collapse, characterized by a
44 strong decrease in the growth yield of the entire population, can occur; this
45 population collapse is also referred in sociomicrobiology as ‘the tragedy of the
46 commons’ [7–11].

47 Although often theorized [5,8], population collapse due to cheater
48 expansion is hard to observe in natural populations even under conditions
49 where cheaters spreading has been observed [12]. This raises the question of
50 how invasion by cheaters is prevented and cooperative behaviors are
51 maintained in microbial populations in nature. Mechanisms such as spatial
52 structure and diffusion [13–22], pleiotropy [10,23–30], restricted migration [31],
53 social and non-social adaptations [11,32–34], policing mechanisms [9],
54 molecular properties of public goods [35], and metabolic strategies [36], have
55 been proposed to play significant roles in maintaining cooperation by preventing
56 cheater invasions and avoiding population collapse [2]. However, cheating
57 behavior is observed *in vitro* [9–11,25], *in vivo* [37,38], and in natural
58 populations, including clinically relevant environments such as the lungs of
59 cystic fibrosis (CF) patients chronically infected with *Pseudomonas aeruginosa*
60 [12,39–45].

61 Importantly, cheater invasion leading to the loss of the cooperative trait
62 and the collapse of the population have been observed in laboratory studies
63 focusing on a single trait [9–11]. However, in environments where more than

64 one social trait is required, the roles among mutants for these traits are likely to
65 be more complex, since a cheater for one trait can be a cooperators for another,
66 making ‘cheater’ and ‘cooperator’ relative terms [46,47]. We hypothesize that, in
67 environments requiring bacteria to express multiple cooperative traits
68 simultaneously, competition among mutants for these traits can influence their
69 social interactions and, therefore, dictate the fate of the population. To test this
70 hypothesis, we examine the consequences of ecological interactions among
71 two social cheaters (mutants for two different traits) and the full cooperators (the
72 wild type) in *P. aeruginosa* populations under conditions where the two
73 cooperative traits are required for optimal growth.

74 Both *lasR* and *pvdS* mutants have been studied individually in a large
75 number of sociomicrobiology studies [10,25,35,48–52], and are among the most
76 common mutants recurrently isolated from the sputum samples of CF patients
77 [12,39,41].

78 LasR is the master regulator of quorum sensing and, among many other
79 genes, controls the production of extracellular elastase [53–55], which is
80 essential for *P. aeruginosa* to use complex sources of amino acids, such as
81 casein, as a carbon and nitrogen source [56–59]. Previous studies showed that
82 *lasR* mutants grow poorly in media containing casein as the only carbon source,
83 but increase in frequency when mixed with wild-type (WT) bacteria. Such
84 increase leads to a population collapse where total cell numbers are drastically
85 reduced due to depletion of producers of the public good [9–11].

86 PvdS is an alternative sigma factor, that among other genes, controls the
87 transcription of genes responsible for pyoverdine biosynthesis [60,61]. In iron-
88 limited environments, *P. aeruginosa* can secrete pyoverdine, which binds iron
89 from the environment and is subsequently retrieved, providing iron to the cell
90 [49]. Mutants in pyoverdine synthesis (e.g. *pvdS*) do not pay the cost of its
91 production but are still able to retrieve the iron-bound pyoverdine produced by
92 others, gaining a fitness advantage and increasing in frequency in mixed
93 populations [35,62,63].

94 We analyzed the cheating of a *lasR* knock-out (KO) mutant over wild-type
95 bacteria in an environment where production of elastase is required (medium
96 with casein as the sole carbon source with iron supplementation). Then, we

97 modified the conditions (medium with casein as the sole carbon source with iron
98 depletion by human apo-transferrin) to cause pyoverdine production to be also
99 required and added a third social player (a *pvdS* KO mutant), to study the
100 interactions in these two different scenarios in short and long-term competition
101 experiments. We found that the fitness advantage of the *lasR* mutant
102 disappears when the *pvdS* mutant is in the culture and the two traits are
103 necessary. The long-term consequence of the interaction between these two
104 mutants is the prevention of the drastic population collapse, which occurs (*i*)
105 irrespectively of the presence of *pvdS* under conditions where only elastase is
106 required, or (*ii*) in the absence of the *pvdS* in conditions where the two traits are
107 required. The observed dynamics can be explained by a simple mathematical
108 model of multiple public good competition, which predicts the dominance of the
109 mutant that avoids expressing the trait with the highest cost, eventually causing
110 the corresponding population collapse associated with the loss of that trait.

111 **Results**

112 ***Cheating capacity of lasR mutant depends on abiotic and biotic factors.***

113 We first investigated the growth yields of the WT and *lasR* and *pvdS*
114 mutants in monocultures under environmental conditions where either trait,
115 both, or none are required. In medium where casein is the sole carbon source,
116 iron-supplemented casein medium (Casein + Fe), and therefore elastase is
117 required, the *lasR* mutant has a lower growth yield than the WT and the *pvdS*
118 mutant (Figure 1A). In medium strongly depleted in iron, as it has been
119 repeatedly shown in the literature, the growth yields of all the three strains,
120 including the WT, are severely reduced [34,35,63–67]. However, the growth
121 yield of the *pvdS* mutant is significantly lower than those of the WT and the *lasR*
122 mutant in iron depleted media with casamino acids (CAA + Transferrin), where
123 only pyoverdine is necessary (Figure 1B). In a medium with casein as the sole
124 carbon source and low iron, namely, iron-depleted casein medium (Casein +
125 Transferrin), where both traits are required, both *lasR* and *pvdS* mutants have a
126 lower growth yield than the WT (Figure 1C). In this medium, the growth yield of
127 the *lasR* mutant is smaller than that of the *pvdS* mutant, indicating that the traits
128 have different benefit/cost ratios in this environment. Importantly, the WT and

129 the two mutants have similar growth yields in a medium where none of the traits
130 are required, iron-supplemented CAA medium (CAA + Fe) (Figure 1D), in
131 accordance with the expectation that the differences in growth yields of the
132 mutants across media are due to the lack of expression of each social trait in
133 the corresponding mutant. Moreover, the observation that the *lasR* mutant only
134 has a growth yield significantly lower than the WT in the media with casein as
135 the sole carbon source indicates that, even though LasR regulates many genes
136 besides those responsible for elastase production [53–55], most do not
137 significantly affect fitness under the conditions tested (For more details about
138 the media used in this study and the growth yield differences of the strains, see
139 Supplemental Information).

140 We then determined the relative fitness of each mutant in competition with
141 the WT and each other in the different media (Figure 2). When co-cultured, at a
142 ratio WT+*lasR* of 9:1, in conditions where only elastase production is required,
143 the *lasR* mutant can cheat on the WT, since *lasR* increases in frequency with
144 respect to it (Figure 2A-left), whereas such increase does not occur when
145 elastase production is not required (Figure 2G). The introduction of the *pvdS*
146 mutant (at ratio WT+*lasR*+*pvdS* of 8:1:1) does not significantly affect the
147 cheating behavior of the *lasR* mutant, since *lasR* can also increase in frequency
148 in the triple co-culture (Figure 2A-right). The incapability of the *pvdS* mutant to
149 affect cheating by *lasR* is consistent with the fact that *pvdS* does not cheat
150 under these conditions (Figure 2B). As expected, *pvdS* can cheat in medium
151 where only pyoverdine is required, whereas *lasR* cannot (Figure 2, panels D
152 and C, respectively). Next, we studied the interaction between these social
153 players in conditions where the two traits are necessary. In these conditions, the
154 *lasR* mutant also increases in frequency in co-culture with the WT (Figure 2E-
155 left). Remarkably, the introduction of the *pvdS* mutant under these conditions
156 causes the cessation of cheating by *lasR* (Figure 2E-right), which is consistent
157 with the fact that *pvdS* can cheat on the WT in co-culture (Figure 2F-left) and
158 both on the WT and the *lasR* mutant in triple co-culture (Figure 2F-right, Figure
159 S1F). Importantly, in conditions where neither of the social traits are necessary,
160 no cheating can be observed (Figure 2G-H), further ratifying that the effects
161 observed are due to social interactions.

162 Notably, *lasR* mutants have been reported to produce lower amounts of
163 pyoverdine than the WT in iron-limited succinate minimal medium [68].
164 However, we found no significant difference in pyoverdine production between
165 WT and *lasR* across the different media used in this study (Figure S2). The
166 difference between our results and those of Stintzi and colleagues might be due
167 to differences in the media used in the two studies [64], or potential differences
168 in the strains used.

169 Altogether, these results demonstrate that the cheating capacities of the
170 two social mutants studied here are context-dependent, varying not only with
171 the environment, but also with the level of polymorphism in the population.

172 ***Invasion of lasR mutant leads to a drastic collapse of the population***

173 We next asked what are the long-term consequences of the different
174 cheating capacities of *lasR* for the overall fitness of the population by
175 performing long-term propagations (Figure 3). We started co-cultures of
176 WT+*lasR* or WT+*lasR*+*pvdS* (at 9:1 and 8:1:1 initial ratios, respectively), either
177 in medium requiring only elastase production (Figure 3A and B), or in medium
178 where the two traits are needed (Figure 3C and D). Propagations were
179 performed by transferring an aliquot of each culture to fresh media every 48
180 hours. Before each passage, growth yields and frequencies of WT, *pvdS*, and
181 *lasR* cells were determined (Figure 3).

182 We observed that, in five out of six replicates of WT+*lasR* co-cultures in
183 the medium where only elastase is required, the *lasR* mutant quickly increased
184 in frequency throughout the first 8 days (4 passages), reaching up to 90% of the
185 population (red bars in Figure 3A). The total cell numbers of the populations
186 (black lines) rapidly decreased by day 12, and no recovery was observed in
187 subsequent passages (Figure 3A). We defined this drastic decrease in density
188 as the population collapse caused by the domination of the *lasR* mutant. One
189 replicate, out of six, did not follow this trend; in this case, no population collapse
190 was observed, and the total cell numbers remained high throughout the
191 experiment (Figure 3A). The fact that this only occurred in one of the six
192 replicates suggests that, in this particular replicate, the WT may have acquired

193 non-social beneficial mutation(s) that could prevent invasion of the *lasR* mutant,
194 as it was described in a recent study [11].

195 Next, we analyzed long-term competitions in triple co-cultures (WT, *pvdS*,
196 and *lasR*; with initial frequencies of 8:1:1, respectively) in the medium where
197 only elastase is required (Figure 3B). In this case, we observed an increase in
198 *lasR* frequency similar to that of seen in WT+*lasR* co-cultures (Figure 3A),
199 which was also accompanied by a drastic decrease in the overall population
200 size. At day 12 of the propagation, all 6 populations collapsed (Figure 3B). The
201 frequencies of the *pvdS* mutant varied between 4% and 15% throughout the
202 experiment, with no indication of any sustained increase (blue bars in Figure
203 3B). This result is consistent with the predictions from the relative fitness
204 measurements, which shows no cheating of *pvdS* in these conditions (Figure
205 2B).

206 Then we propagated WT+*lasR* co-cultures in the medium where the two
207 traits are necessary (Figure 3C). In these propagations, the *lasR* mutant also
208 increases in frequency throughout the first days, but at a slower pace than when
209 only elastase is required (compare panels A and C in Figure 3). The total cell
210 numbers remain high until days 10-12, but, as the *lasR* frequencies increase to
211 about 80%, the density of the population decreases, collapsing by day 18.
212 Hence, in all the three scenarios described above, the dominance of the *lasR*
213 mutant, which presumably resulted in the exhaustion of the public good
214 elastase, caused a drastic population collapse (Figure 3A–C).

215 ***pvdS* prevents the drastic population collapse caused by the invasion of** 216 ***the lasR* mutant**

217 Our short-term competitions revealed that the cheating capacity of *lasR* is
218 influenced not only by abiotic, but also by biotic conditions, as the presence of
219 *pvdS* under conditions where both traits are required reduces the relative fitness
220 of the *lasR* mutant (Figure 2C). Therefore, we investigated if, in propagations in
221 the medium where both traits are needed, *pvdS* could prevent the drastic
222 population collapse caused by *lasR* invasion. Indeed, Figure 3D shows that
223 *lasR* does not increase in frequency, staying at approximately 3% throughout
224 the experiment. In contrast, *pvdS* rapidly expands to an average frequency of

225 96% at day 18. As the *lasR* mutant does not increase in frequency, cell
226 densities of the overall populations do not decrease and collapse of the
227 population is not observed.

228 Given that *pvdS* dominated the populations, a reduction in cell numbers
229 due to its invasion could be expected. Indeed, full fixation of the *pvdS* mutant
230 should result in a small population decrease close to the levels of the *pvdS*
231 mono-cultures (Figure 1C). However, at the end of the propagation experiments
232 (day 18), complete fixation of *pvdS* had not yet been reached, and an average
233 of 4% of WT and *lasR* cells were detected in the populations (Figure 3D). We
234 hypothesized that the presence of only 4% of pyoverdine producers in the
235 population could be enough to sustain the growth of the entire populations to
236 levels similar to the WT mono-cultures similarly to what has been reported
237 recently for cultures in chemostat [69]. The results shown in Figure S3A support
238 this hypothesis, since the growth yields of WT+*pvdS* mixed cultures at different
239 initial frequencies of *pvdS* significantly decrease only when the initial frequency
240 of *pvdS* is 98% or less, whereas mixtures with 3-4% of WT cells (or WT and
241 *lasR* cells) have growth yields similar to that of WT monocultures. These results
242 demonstrate that a small proportion of pyoverdine producer cells (WT and/or
243 *lasR* cells) are sufficient to produce enough pyoverdine to sustain the entire
244 population. This justifies why, in the propagation shown in Figure 3D, where at
245 day 18, *pvdS* reached an average frequency of 96%, no significant drop in cell
246 numbers was observed. Moreover, these results indicate that, if the
247 propagations were to continue, *pvdS* fixation and the subsequent decrease in
248 cell density could be expected. In fact, new WT+*pvdS* and WT+*pvdS*+*lasR*
249 propagations with much higher initial frequencies of *pvdS* (75-85%), allowed to
250 observe this population collapse (Figure S3B and C). The reason why *lasR* or
251 *pvdS* domination lead to a stronger or milder population collapse, respectively,
252 is related with the different characteristics of these two mutants, which have
253 different fitness in mono-culture in the medium requiring both traits (Figure 1C),
254 presumably as a consequence of the differences in cost and benefits of the
255 traits involved.

256 Remarkably, the presence of the *pvdS* mutant in the 3-way competition
257 under conditions where the two traits are required has a strong effect on the

258 outcome of the propagations in terms of growth yields, which is dramatically
259 different from those of the other three scenarios tested, since *pvdS* domination
260 prevents the drastic population collapse (Figure 3D) caused by the expansion of
261 *lasR* in the other three conditions (Figure 3A–C). This occurs because, in this
262 environment, although the *lasR* mutant is still being able to cheat on the WT
263 (Figure S1C), it is being cheated by *pvdS* (Figure S1D).

264 Importantly, long-term propagation experiments of WT+*lasR* and
265 WT+*lasR*+*pvdS* in medium where neither of the traits are required showed no
266 significant change in the population densities (Figure S4).

267 ***Alterations in carbon or iron source availability can prevent or induce the*** 268 ***collapse***

269 We reasoned that, if social interactions dominate over *de novo* adaptive
270 mutations in long-term dynamics, alterations of the abiotic factors in the triple
271 cultures should modify the social role of each mutant (by changing the costs
272 and benefits of the cooperative traits) and therefore affect the outcome for the
273 populations. Indeed, in the propagation of WT+*lasR* co-cultures in the medium
274 where only elastase is required, changing the carbon source from casein to
275 CAA during the course of the propagation (making elastase unnecessary)
276 eliminates the advantage of the *lasR* mutant, and this environmental change is
277 sufficient to prevent the population collapse (Figure 4A). Conversely, addition of
278 iron to the medium where both traits are required (thus making pyoverdine
279 unneeded) reverts the expansion of the *pvdS* mutant, favoring *lasR* cheating,
280 and ultimately causing the collapse of the populations at day 18 (Figure 4B). We
281 confirmed that changes in final frequencies observed in Figure 4B were not due
282 to the high initial frequencies of *pvdS*, because even though the selective
283 advantage of *pvdS* is frequency dependent, this mutant is capable of cheating
284 even at frequencies higher than 90% (Figure S5).

285 Overall, these results confirm the cheating role of the two mutants, and
286 also demonstrate the preponderance of social interactions over evolutionary
287 adaptation by *de novo* mutation in the propagation experiments shown in Figure
288 3.

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

291 To further investigate the general factors determining the dynamics of
292 competitions among cooperators and cheaters we built a simple mathematical
293 model (see Supplemental Information, Mathematical Model 1 and 2). The model
294 assumes that the cost (c) of a cooperative trait is lower than the benefit (b)
295 associated with this trait ($b > c > 0$), and also that the benefit provided by the
296 cooperative trait is equal for the entire population, as it would be expected in the
297 case of an equally accessible public good in a well-mixed environment. Spatial
298 structure, diffusion, or privatization, which would alter the benefit gained from
299 the public good for cooperators and cheaters asymmetrically, were not
300 considered in the model. The parameters used are described in Supplementary
301 Table S1. As can be seen from the fitness definitions of the three players
302 involved in our simple 3-way public model (Supplemental Information,
303 Mathematical Model 1 (equations 1 to 3)), the cheaters always have a higher
304 fitness than the cooperator due to the costs (c_1 or c_2) saved. Figure 5A shows
305 the predicted mean fitness (\bar{w}) and final frequencies of the different strains in
306 the population assuming different c_1/c_2 ratios. It can be easily seen that
307 cooperators will always go extinct, and that the two cheaters can only co-exist
308 when $c_1 = c_2$. Whenever $c_1 \neq c_2$, then the cheater that produces the trait with
309 highest cost will lose. Therefore, the relation between c_1 and c_2 determines
310 which cheater will dominate the population, independently of the benefits (b_1
311 and b_2) of these cooperative traits. On the other hand, the mean fitness, \bar{w} , is
312 affected by the difference between b and c values of each trait.

313 We simulated the four scenarios corresponding to the conditions in Figure
314 3. As shown in Figure S6 in panels A and C, the cooperator for both traits and
315 the cheater of the 1st cooperative trait compete, while the cheater of the 2nd
316 cooperative trait is absent, whereas in panels B and D all three strains compete.
317 In panels A and B, only the 1st cooperative trait is necessary, while in panels C
318 and D both traits are required. The c_1/c_2 ratios defined in the model are
319 estimated from the ratios of the relative fitnesses determined in the competitions
320 shown in Figure 2E and F. The results of the model for the four scenarios
321 resemble the experimental data, explaining changes in frequencies reasonably

322 well (Figure S6A-D). However, this simple model predicts complete fixation of
323 the expanding mutant (Figure S6A-D), and cannot explain the lack of fixation of
324 *lasR* (Figure 3A–C) or *pvdS* (Figure 3D) observed experimentally. As discussed
325 above, as long as *de novo* mutations are not acquired, *pvdS* can reach fixation
326 when co-cultured either with WT, or with WT and *lasR* under conditions where
327 the two traits are needed (Figure S3B and C), which is in accordance to the
328 model. However, that was not the case when *lasR* expansion was observed.
329 We tested experimentally whether fixation of the *lasR* mutant could occur if the
330 propagations were continued under conditions where *lasR* was expanding. Our
331 results show that, when we initiate WT+*lasR* competitions at initial *lasR*
332 frequencies similar to those at day 18 in Figure 3A, *lasR* still fails to reach
333 fixation (Figure 6A). This dynamical behavior of *lasR* is not predicted under the
334 assumptions of the model and suggests that other processes are taking place in
335 the experiment.

336 Given that the *lasR* gene and elastase production are regulated by quorum
337 sensing, we hypothesized that quorum sensing could be responsible for the lack
338 of fixation of *lasR* mutant observed experimentally. Quorum sensing regulation
339 should reduce both the cost and the benefit of elastase production when the
340 cooperators are below the quorum sensing threshold, as cells will not produce
341 elastase in that phase. We therefore modelled the effect of quorum sensing on
342 fitness equations by assuming a Hill function where the cost and benefit of the
343 1st cooperative trait are sharply reduced when the frequency of the cheater for
344 the 1st cooperative trait reaches a given threshold value (see Supplemental
345 Information, Mathematical Model 2 for the model including quorum sensing). In
346 this case, fixation of the mutant for the 1st cooperative trait can only happen if
347 $c_1 < c_2$. When $c_1 \geq c_2$, both cheaters can co-exist in the population (Figure 5B). As
348 shown in Figure 7, the simulations of the modified model including quorum
349 sensing for the four experimental conditions predict accurately their frequency
350 dynamics. Moreover, it also predicts that mutants for traits regulated by quorum
351 sensing, like *lasR*, will not reach fixation.

352 To test experimentally if quorum sensing regulation could indeed be the
353 mechanism responsible for preventing fixation of *lasR* in the WT+*lasR*
354 competitions, we repeated the propagation experiment shown in Figure 6A

355 adding the quorum sensing autoinducer Acyl-homoserine lactone (AHL) N-3-
356 oxododecanoyl-homoserine lactone (3OC₁₂-HSL) to the culture medium.
357 Addition of AHL abolishes the quorum sensing-dependent regulation of
358 elastase, locking elastase production constitutively in the ON state.
359 Remarkably, the addition of AHL allows the *lasR* mutant to expand throughout
360 the competitions, and eventually reach fixation (Figure 6B), as the model
361 without quorum sensing predicted (Figure 5A). Thus, regulation of the
362 production of a public good by quorum sensing prevents full domination of the
363 quorum sensing cheater, maintaining cooperation in populations. However, if
364 the expanding cheater is affected in the production of a public good not
365 regulated via quorum sensing (e.g. *pvdS*), this mutant can dominate the entire
366 population.

367 In summary, the results obtained with our 3-way public goods model
368 including quorum sensing (Figure 7) show that the dynamics observed in our
369 propagation experiments (Figure 3) can be explained by the relationship
370 between the cost of the different cooperative traits involved and a quorum
371 threshold that regulates both costs and benefits of one of these traits. Figures
372 S6E-I represent predictions, according to our model, for other possible
373 scenarios with different relationships between the costs, which can be tested
374 experimentally in the future (see Supplemental Information).

375 Discussion

376 The classical experimental approach in sociomicrobiology has been to
377 study one trait at a time. The simplicity of such an approach has allowed to
378 substantially increase our understanding of the dynamics of cooperative
379 interactions and revealed several mechanisms involved in the maintenance of
380 cooperation [2,4,47]. In particular, the ability of *lasR* or *pvdS* mutants to behave
381 as cheaters individually has been extensively documented [9,11,25–
382 27,34,35,64,67,70–74], and these mutants are commonly isolated from bacterial
383 populations colonizing CF lungs [41,45].

384 We established an experimental setup where WT cooperates in more than
385 one trait: production of elastase via quorum sensing regulation and production
386 of siderophore pyoverdine. Under conditions where the two traits are required,

387 the *lasR* mutant can act a cheater for elastase but a cooperators for pyoverdine,
388 whereas the *pvdS* mutant can cooperate for elastase production and cheats for
389 pyoverdine production. Our results showed that, in this environment, the 3-way
390 competitions result in a dominance of *pvdS* over both the WT and the *lasR*
391 mutant. Presumably, this occurs because the advantage of the *pvdS* mutant
392 (caused by not producing pyoverdine) is higher than that of *lasR* (for not
393 producing elastase, and the other quorum sensing regulated goods) under
394 conditions where the two traits are necessary, causing *pvdS* to be more fit than
395 *lasR* in this environment (Figure 1C). As a consequence, the *pvdS* mutant can
396 cheat on the *lasR* mutant (and on the WT) (Figure 2, Figure S1), dramatically
397 affecting the outcome of the long-term competitions (Figure 3D).

398 The expansion of *pvdS* under conditions where the two traits are required
399 prevents the drastic population collapse caused by invasion of *lasR* mutants
400 observed in absence of *pvdS* (compare panels D and C of Figure 3). Even
401 though the domination of *pvdS* mutant can also lead to a drop in the density of
402 the population caused by the exhaustion of the public good, (Figure S3B and
403 C), the decrease in cell density due *pvdS* domination is much less drastic than
404 that of observed upon domination of *lasR* mutant (Figure 3A – C).

405 Interestingly, both *lasR* and *pvdS* mutants are stronger cheaters in the
406 medium where either of their affected trait is required than in the medium where
407 the two traits are necessary (Figure 2, panels A and D versus E and F). In the
408 case of *lasR*, this difference is coherent with the lower growth yields reached
409 under conditions where the two traits are required (Figure 1, A versus C), which
410 allow fewer cell divisions and therefore milder cheating. The difference in
411 cheating of *pvdS* cannot be ascribed to higher growth yields when only
412 pyoverdine is required (Figure 1B and C). However, pyoverdine production per
413 cell is significantly higher in the medium where only pyoverdine is required
414 (Figure S2G-H), and this could possibly explain the boosting in the cheating by
415 *pvdS* in this medium. A stronger iron depletion in the CAA + Transferrin medium
416 is coherent with both the low iron content of the CAA mixture used in our
417 experiments [75] and the iron-chelating capacity of casein [76,77], which could
418 allow carryover of casein-bound iron to the medium and thus result in higher
419 iron availability in the media with casein.

420 Our simple model assuming that the difference in costs and benefits of the
421 cooperative traits involved proved to be sufficient to reasonably explain our
422 experimental results. Therefore, the model allowed us to infer the general
423 parameters governing social interactions beyond the particularities of the two
424 mutants used in this study. Specifically, the mathematical model suggests that,
425 in competitions among more than one social cheater under conditions where
426 more than one trait is required (a scenario likely to be closer to the conditions in
427 nature), the mutant for the trait with the highest cost is expected to dominate.
428 Moreover, the degree of the decrease in population density caused by loss of
429 cooperation due to exhaustion of the public good is determined by the benefit
430 minus the cost difference of the trait affected. In case of a trait with high benefit-
431 cost difference, a drastic collapse on the density of the population caused by
432 the cheater in that trait is expected. In contrast, if the mutant for the trait with a
433 low benefit-cost difference (as inferred for *pvdS*) dominates, a weak drop
434 occurs. These scenarios that lead to different degrees of decrease in population
435 densities could have very different consequences for the host in the context of
436 infections.

437 Importantly, our results provide support for a dynamic view of cooperation
438 and cheating that is dependent on both the genotypes present in the population
439 and the environmental conditions. We demonstrated how changes in the abiotic
440 environment can cause a social mutant to start or stop cheating or being
441 cheated. Additionally, as shown here for the *lasR* mutant, quorum sensing
442 regulation can also favor the maintenance of polymorphism, since such
443 regulation alters the values of the cost and benefits of the traits as a function of
444 the population density.

445 A better understanding of the interactions in polymorphic bacterial
446 populations in complex environments not only helps to gain insights into key
447 aspects of sociomicrobiology, but also can provide a theoretical framework for
448 the development of new therapeutic strategies against bacterial populations
449 where social mutants can invade [41,45]. In particular, our study provides
450 relevant information about the biotic and abiotic conditions that favor the
451 expansion of these mutants, which should be taken into account when

452 considering strategies aiming to manipulate populations where this type of
453 social interactions is taking place.

454 The potential effects of the appearance of *pvdS lasR* double mutants in
455 settings similar to ours should also be considered since double mutants have
456 the potential to occur *in vivo* [41]. Although we found no evidence for
457 emergence of *pvdS lasR* double mutants within the period of the experiments
458 reported here, in the course of longer propagations *pvdS lasR* double mutants
459 generated by *de novo* mutations were identified (data not shown). The effects of
460 these double mutants on the interactions described here should be investigated
461 in the future. However, based on our results, we can speculate that double
462 mutants, as full cheaters, should cause an accelerated collapse of the
463 population.

464 A non-social explanation for the advantage of the *pvdS* mutant in triple co-
465 cultures under conditions where the two traits are required was also considered
466 given that, at least in *Pseudomonas fluorescens*, certain mutants defective in
467 pyoverdine production have been reported to be better adapted even in
468 environments where iron concentration is not low, and thus can be considered
469 non-social mutations [78]. However, the fact that our *pvdS* mutant has a lower
470 fitness than the WT in the low iron media and does not show any advantage in
471 conditions where pyoverdine production is not necessary (Figure 1B and C,
472 Figure 2B and H) rules out non-social adaptation as the reason for its
473 advantage.

474 Collectively, our findings underline the need for including polymorphism in
475 social phenotypes and multiple environmental conditions in experimental
476 studies and mathematical models pertaining to cooperation in microbial
477 populations. This need is further supported by recent theoretical and
478 experimental studies showing that interactions between genetically and
479 functionally interlinked cooperative traits can significantly affect the course of
480 their social evolution [26,79]. Our results demonstrate that using experimental
481 conditions that include more than one social trait can reveal complex and
482 dynamic social roles in bacterial populations as well as their dependence on the
483 environment. Understanding the dynamics of polymorphic populations in these
484 complex environments provides insights into social interaction processes,

485 expanding their relevance beyond sociomicrobiology, in addition to providing
486 important knowledge for the development of novel therapeutic tools.

487 **Materials and Methods**

488 **Bacterial strains.** The strains used in this study were *Pseudomonas*
489 *aeruginosa* WT strain PA01, PA01 *lasR* mutant harboring a gentamycin
490 resistant gene inserted in *lasR* (*lasR::GmR*) [80], and PA01 *pvdS* mutant
491 harboring a gentamycin resistance gene replacing the *pvdS*
492 coding sequence ($\Delta pvdS::GmR$) [81]. For more detailed information, see
493 Supplemental Information, supplementary methods section.

494 **Media and culture conditions.** The medium where only elastase is required
495 (iron-supplemented casein medium) contains casein (Sigma, Ref: C8654) (1%
496 w/v) as the sole carbon and nitrogen source salts (1.18 g $K_2HPO_4 \cdot 3H_2O$ and
497 0.25 g $MgSO_4 \cdot 7H_2O$ per liter of dH_2O) and 50 μM of $FeCl_3$. The medium where
498 only pyoverdine production is required (iron-depleted CAA medium) contains
499 the same salt solutions indicated above, low iron CAA (BD, Ref: 223050) (1%
500 w/v) as the sole carbon source and 100 $\mu g/ml$ of human apo-transferrin (Sigma,
501 T2036) and 20 mM sodium bicarbonate to deplete available iron and induce
502 pyoverdine production. The medium where both traits are needed (iron-depleted
503 casein medium) is identical to the iron-supplied casein medium but instead of
504 $FeCl_3$ supplementation, this medium contains 100 $\mu g/ml$ of human apo-
505 transferrin (Sigma, T2036) and 20 mM sodium bicarbonate to deplete available
506 iron and induce pyoverdine production. The medium where none of the traits is
507 necessary (iron-supplemented CAA medium) contains the same salt solutions
508 as the other media, low iron CAA (1% w/v) as the sole carbon source and 50
509 μM of $FeCl_3$. All cultures were incubated in 15 ml falcons at 37°C with aeration
510 (240 rpm, New Brunswick E25/E25R Shaker) for the incubation times indicated.
511 Cell densities were estimated by measuring absorbance (Abs) at 600 nm
512 (OD_{600}) in a Thermo Spectronic Helios δ spectrophotometer.

513 **Determination of genotypic frequencies.** Estimation of the frequencies of
514 each strain in the co-cultures was performed by scoring fluorescence and
515 colony morphology of colonies obtained from plating serial PBS dilutions of the
516 cultures. For each individual sample, three aliquots (of 50 μl - 200 μl , as

517 appropriated) were plated into LB agar plates, which were used as technical
518 replicates. Then, CFU/ml were calculated by scoring different colony
519 morphologies on each plate (with three technical replicate for each biological
520 replicate). A stereoscope (Zeiss Stereo Lumar V12) with a CFP filter was used
521 to distinguish pyoverdine producers, which are fluorescent, from the non-
522 fluorescent *pvdS* mutants [72,82]. *lasR* mutant colonies have distinct colony
523 morphology: smaller with smooth edges whereas elastase producers are larger
524 with rugged edges [82]. To validate the phenotypic scoring all colonies used to
525 determine the frequency from day 18 of the propagation experiments (Figure
526 3D) were tested by PCR with primers for the *lasR* and *pvdS* genes. The PCR
527 data confirmed the phenotypic scoring with 100% accuracy.

528 **Measurement of relative mutant fitness.** Relative fitness was used to
529 determine the cheating capacity of each mutant as commonly used [72,83,84].
530 For both mutants (*lasR* and *pvdS*), the relative fitness of each mutant (v) was
531 calculated as the change in frequency of the mutant over a period of 48 hours
532 relative to the rest of the strains in the mixture, *i. e.*, $v = fm_{final} \cdot fr_{initial} / fm_{initial} \cdot fr_{final}$
533 [72,83,84]. Where fm is the proportion of the mutant measured at the beginning
534 of the competitions for $fm_{initial}$, or after 48 hours of competition for fm_{final} , and fr is
535 the final proportions of the rest of the strains in the competitions at time = 0
536 ($fr_{initial}$) or after 48 hours (fr_{final}). As $fr = (1 - fm)$, the relative fitness was
537 determined using the following formula $v = fm_{final} (1 - fm_{initial}) / fm_{initial} (1 - fm_{final})$.

538 **Competition experiments.** We propagated six replicates under four different
539 conditions (Figure 3). Prior to start the competition experiments, all strains were
540 inoculated, from frozen stocks, in medium containing 1% (w/v) casein and 1%
541 (w/v) CAA in salts solution (same as in iron-supplied casein medium, described
542 above) for 36 hours at 37°C temperature with shaking (240 rpm). Cells were
543 then washed with PBS four times, to remove any residual extracellular factor.
544 Next after measuring cell densities (OD₆₀₀), cultures were normalized to OD₆₀₀ =
545 1.0 and used to inoculate the various media as described in the text and figures.
546 The different strains were diluted into fresh media, at different ratios as
547 specified, to a starting initial OD₆₀₀ = 0.05. For short term competitions the
548 relative frequencies were determined by plating an aliquot of each culture at the
549 beginning of the experiment (t = 0), and after 48 hours of incubation. For long-

550 term competitions, the relative frequencies were determined at $t = 0$, and
551 thereafter every 48 hours before each passage. At the end of every 48 hours
552 1.5 μ l of each culture was transferred to 1.5ml of fresh medium (bottle-neck of
553 1/1000).

554 **Statistical analysis.** Independent biological replicates were separately grown
555 from the frozen stocks of each strain. Each figure (or figure panel) includes data
556 from at least 6 biological replicates. The sample size (N), corresponds to the
557 total numbers of independent biological replicates in each figure panel and is
558 provided in the corresponding figure legends. The Mann-Whitney test which is a
559 non-parametric test, was used because it does not account for normality and it
560 is more suitable for the sample size used in each experiment ($5 < N < 20$). For
561 multiple corrections, Kruskal-Wallis test with Dunn's correction was used. For all
562 statistical analyses we used GraphPad Prism 6 software
563 (<http://www.graphpad.com/scientific-software/prism>).

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577 **Author Contributions**

578 Conceptualization, Ö.Ö., K.X., I.G., and R.B.; Methodology, Ö.Ö., K.X.,
579 I.G., and R.B.; Investigation, Ö.Ö.; Writing – Original Draft, Ö.Ö. and R.B.;

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582 Declaration of Interest

583 The authors declare no competing interests.

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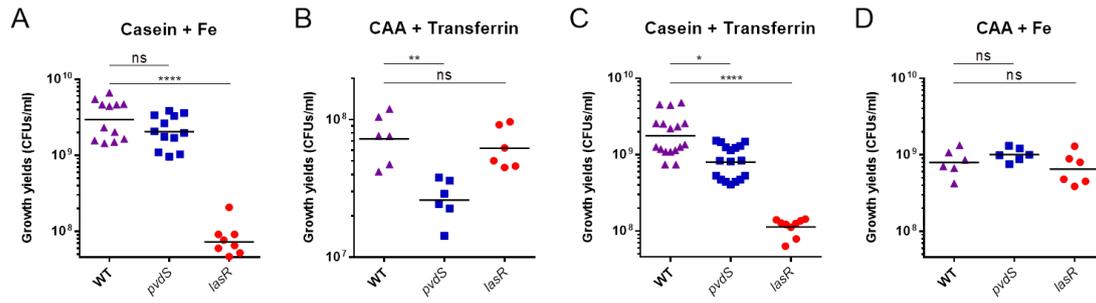


Figure 1. *P. aeruginosa lasR* and *pvdS* mutants have lower growth yields than WT in media where elastase and/or pyoverdine are required, respectively. Growth yields (CFU/ml) of WT (purple triangles), *pvdS* (blue squares), and *lasR* (red circles) strains of *P. aeruginosa* monocultures after 48 hours of incubation in (A) iron-supplemented casein medium (Casein + Fe), (B) iron-depleted casamino acids medium (CAA + Transferrin), (C) iron-depleted casein medium (Casein + Transferrin) and (D) iron-supplemented casamino acids medium (CAA + Fe). Each data point represents an individual biological replicate (N \geq 6) and the horizontal bars indicate the means of each group. For comparisons, Kruskal-Wallis test with Dunn's correction was used, ns=not significant P>0.05, * P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001, **** P \leq 0.0001.

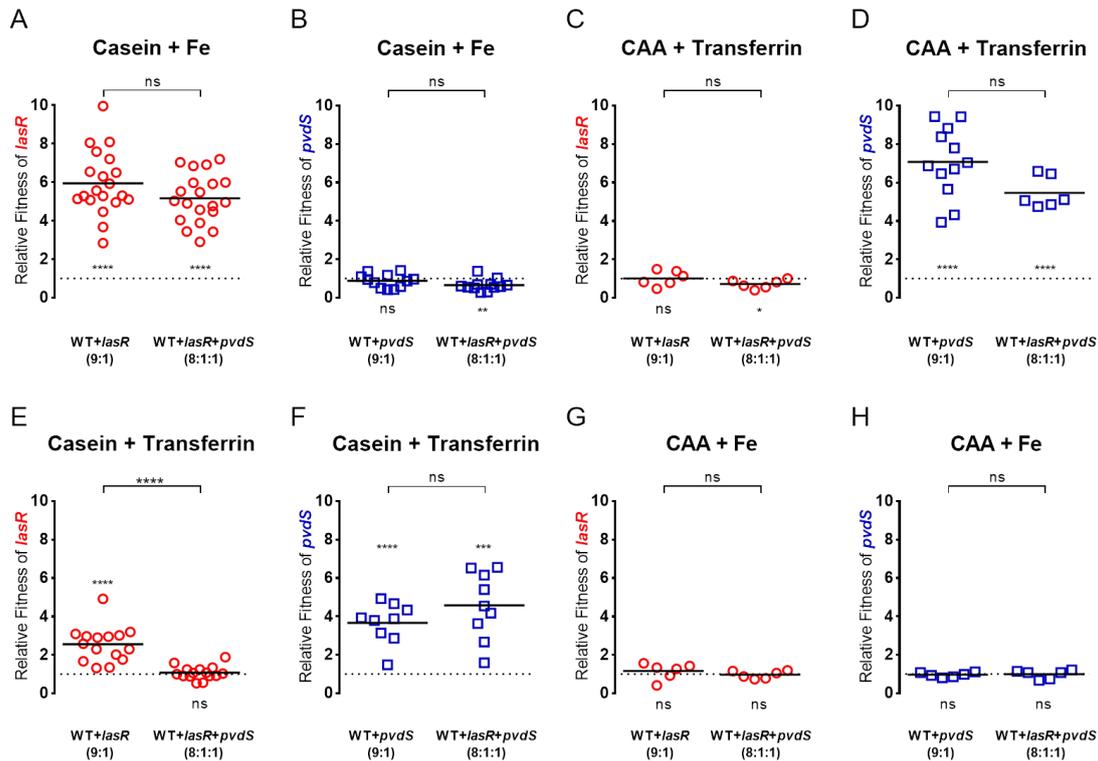


Figure 2. Relative fitness of *lasR* or *pvdS* in various media in double or triple co-cultures.

Relative fitness (v) of *lasR* (red circles in A, C, E, and G) or *pvdS* (blue squares in B, D, F, and H) were calculated as the change in frequency of each mutant relative to the rest of the strains in each culture after 48 hours of incubation in (A and B) iron-supplemented casein medium (Casein + Fe), (C and D) iron-depleted casamino acids medium (CAA + Transferrin), (E and F) iron-depleted casein medium (Casein + Transferrin), and (G and H) iron-supplemented casamino acids medium (CAA + Fe). Relative fitness of *lasR* (A, C, E, and G) was calculated in co-cultures with WT, or with WT and *pvdS*. Relative fitness of *pvdS* (B, D, F, and H) was calculated in co-cultures with WT, or with WT and *lasR*. 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*. Mann-Whitney two-tailed test was used to compare the relative fitness values of each mutant in double and triple co-cultures (significance symbols are located at the middle-top of each plot above the brackets). Dotted lines indicate $v=1$. Relative fitness values above the dotted lines ($v>1$) indicate that the strain is cheating and below the dotted lines ($v<1$) indicate that the strain is being cheated. One-sample t-test was used to determine whether each dataset is significantly different than 1 (significance symbols are located above the dotted line when $v>1$ and below the dotted line when $v\leq 1$). Each data point indicates an individual biological replicate ($N\geq 6$) and horizontal lines indicate the means of each group. ns=not significant $P>0.05$, * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$, **** $P\leq 0.0001$.

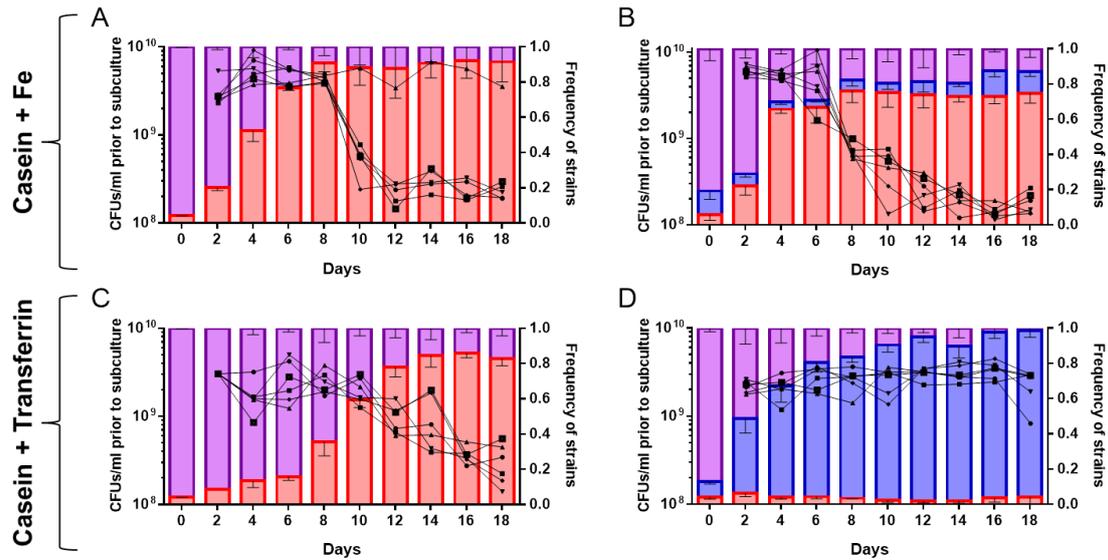


Figure 3. Effects of abiotic and biotic factors on growth yields and strain composition of the population in long-term propagations. Left Y-axes represent CFU/ml values prior to subculture; black symbols correspond to the CFU/ml values of each of the 6 biological replicates tested for each condition. Right Y-axes show the frequencies of WT (purple), *lasR* (red), and *pvdS* (blue) at each time point; data are shown as bars and represent the means of 6 biological replicates, error bars indicate SD. X-axes show the days of propagations. **(A)** WT and *lasR* co-cultures mixed at an initial frequency of 9:1 in iron-supplemented casein media. **(B)** WT, *lasR*, and *pvdS* triple co-cultures mixed at initial an initial frequency of 8:1:1 in iron-supplemented casein media. **(C)** and **(D)** same as in (A) and (B) but in iron-depleted casein media.

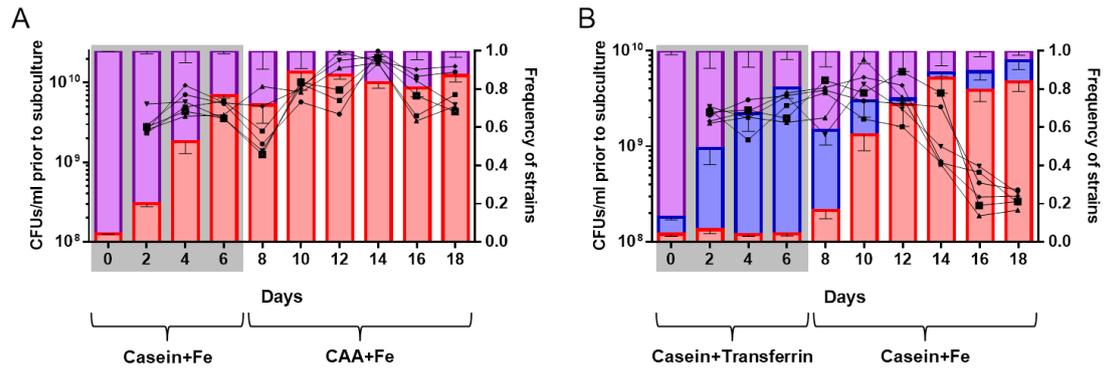


Figure 4. Effects of alterations of abiotic conditions in long-term propagations. (A) After the 6th day of the competitions of WT+*lasR* co-cultures in iron-supplemented casein medium (Casein + Fe) (Figure 3A), aliquots were transferred into iron-supplemented CAA medium (CAA + Fe) to relieve the requirement for digesting casein by elastase production (N=6, data from the first 6 days are from Figure 3A). **(B)** After the 6th day of the competitions of WT+*lasR*+*pvdS* triple co-cultures in iron-depleted casein medium (Casein + Transferrin) (Figure 3D), aliquots were transferred into iron-supplemented casein medium (Casein + Fe) to relieve the requirement for pyoverdine production (N=6, data from the first 6 days are from Figure 3D). Legends as in Figure 3.

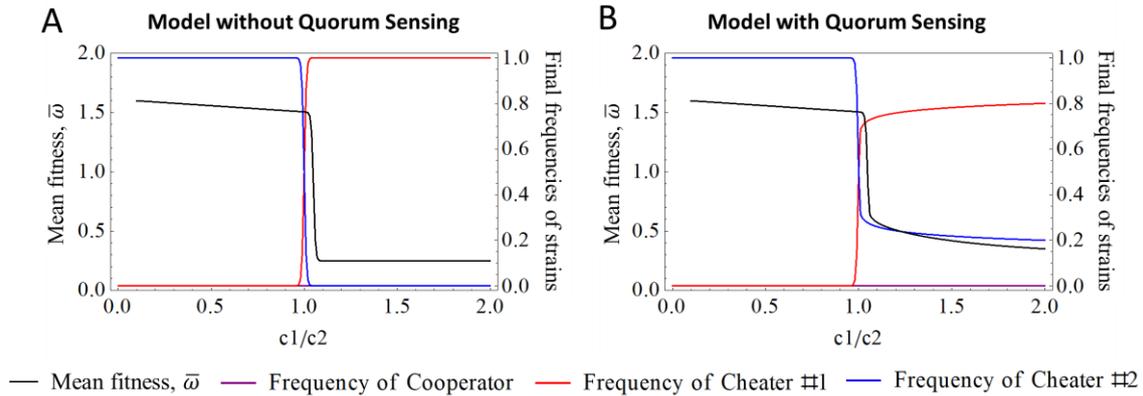


Figure 5. Mathematical model for the final frequencies of the three strains in relation to the ratio of c_1/c_2 . In Left-Y axes, the mean fitness, $\bar{\omega}$, is shown in black. In Right-Y axes, frequencies of cheater of the 1st cooperative trait (red), cheater of the 2nd cooperative trait (blue),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

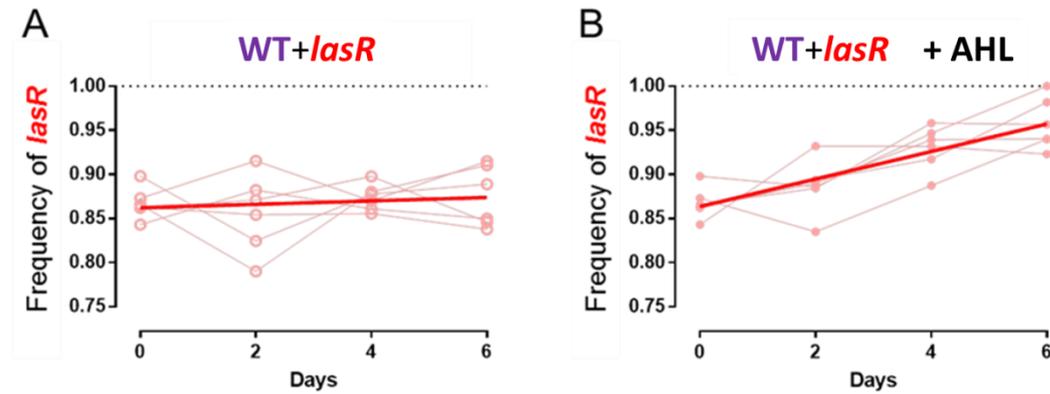


Figure 6. Frequencies of *lasR* in propagations of WT+*lasR* co-cultures in iron-supplemented casein media in the absence or presence of exogenously added quorum sensing signal AHL. Initial frequencies of *lasR* of 80-90% were used, these frequencies were similar to those of the 18th day in Figure 3A. 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 μ M AHL (3OC₁₂-HSL) added to the media. Red lines indicate linear regressions. Dotted lines represent 100% domination of *lasR*.

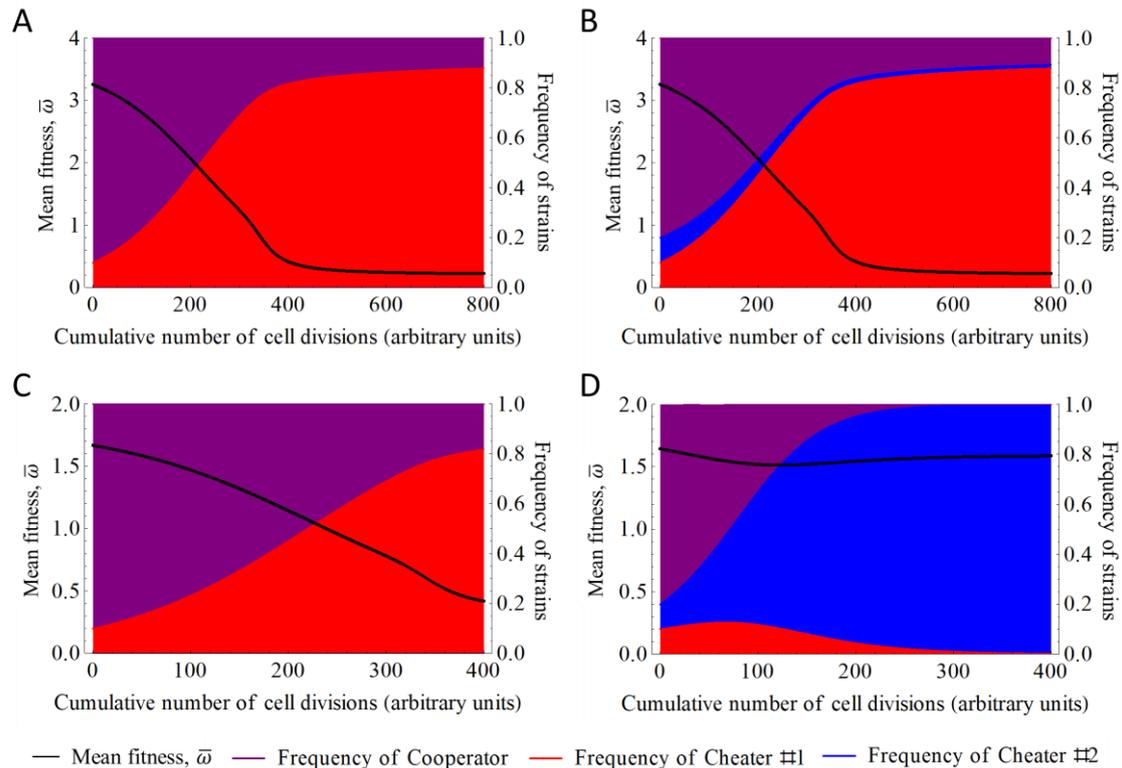


Figure 7. Results of the mathematical model simulating the four scenarios in Figure 3. Model includes quorum sensing regulation of the 1st cooperative trait (b_1 and c_1 are negatively regulated via a Hill equation as a function of the frequency of the mutant of this trait, p_{ch1}). Left Y-axes show $\bar{\omega}$, the mean fitness of the entire population which is a function of b and c values; these values correspond to the biomass gain due to benefiting from the cooperative action (b), and the energy spent to the cooperative action instead of biomass increase (c). 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 cumulative numbers of cell divisions as arbitrary units. In panels (A) and (B), only the 1st cooperative trait is necessary ($b_1 > c_1 > 0$, whereas $b_2 = c_2 = 0$), while in panels (C) and (D) both traits are required ($c_2 > c_1 > 0$ and $b_1 > b_2 > 0$). In panels (A) and (C), the cooperators for both traits (WT) and the cheater of the 1st cooperative trait compete ($p_{coop}(0) = 0.9$ and $p_{ch1}(0) = 0.1$), while the cheater of the 2nd cooperative trait is absent ($p_{ch2}(0) = 0$), whereas in panels (B) and (D) all three strains compete ($p_{coop}(0) = 0.8$ and $p_{ch1}(0) = p_{ch2}(0) = 0.1$). 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) $p_{coop}(0) = 0.8$, $p_{ch1}(0) = 0.1$, $p_{ch2}(0) = 0.1$, $c_1 = 0.01$, $b_1 = 1.5$, $c_2 = 0.025$, $b_2 = 0.25$, $\omega_0 = 0.1$, $th = 0.8$, $n = 30$. Note that the values of parameters used in these simulations are chosen to reflect approximately the relation between the values observed in Figure 1, Figure 2. (For more detailed description please see Supplemental Information, Mathematical Model 2).