Adaptation to public goods cheats in *Pseudomonas aeruginosa*

Siobhán O'Brien[1]*, Adela M. Luján[2], Steve Paterson[3], Michael A. Cant[4] & Angus Buckling[5]

[1]Center for Adaptation to a Changing Environment (ACE), ETH Zürich, 8092 Zürich, Switzerland. siobhan.obrien@env.ethz.ch

[2] College of Life and Environmental Sciences, University of Exeter, Penryn Campus. Cornwall TR10 9FE, United Kingdom

[3] Centro de Investigaciones en Química Biológica de Córdoba, CIQUIBIC, CONICET and Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA, Córdoba, Argentina

[4]Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, UK, L69 7ZB

[5]Environment and Sustainability Institute, University of Exeter, Penryn Campus, TR10 9FE, United Kingdom

**Corresponding author*

Abstract

Cooperation in nature is ubiquitous, but is susceptible to social cheats who pay little or no cost of cooperation yet reap the benefits. The effect such cheats have on reducing population productivity suggests that there is selection for cooperators to mitigate the adverse effects of cheats. While mechanisms have been elucidated for scenarios involving a direct association between producer and cooperative product, it is less clear how cooperators may suppress cheating in an anonymous public goods scenario, where cheats cannot be directly identified. Here, we investigate the real-time evolutionary response of cooperators to cheats when cooperation is mediated by a diffusible public good: the production of iron-scavenging siderophores by *Pseudomonas aeruginosa*. We find that siderophore producers evolved in the presence of a high frequency of non-producing cheats were fitter in the presence of cheats, at no obvious cost to population productivity. A novel morphotype independently evolved and reached higher frequencies in cheat-adapted versus control populations, exhibiting reduced siderophore production but increased production of pyocyanin - an extracellular toxin that can also increase the availability of soluble iron. This suggests that cooperators may have mitigated the negative effects of cheats by downregulating siderophore production and upregulating an alternative iron-acquisition public good. More generally, the study emphasises that cooperating organisms can rapidly adapt to the presence of anonymous cheats without necessarily incurring fitness costs in the environment they evolve in.

Keywords: Siderophore; Public goods; Cooperation; Pseudomonas; Pyoverdine; Experimental Evolution

Background

Cooperative behaviour (any action selected at least partly because of its beneficial effect on another individual [1]) owes its ubiquity to incurring a direct or indirect fitness benefit to individuals in cooperating groups. However, when cooperation carries a cost it is associated with social cheats who pay little or no cost of cooperation but reap the rewards. This fitness advantage facilitates their invasion of cooperating groups, which can impose a large cost on the population as a whole, in a 'Tragedy of the Commons' scenario [2,3]. However, cooperation persists at all levels of biological organisation [4], suggesting that mechanisms have evolved that impede the negative impact of cheats. Many organisms, including long-tailed tits [5], bumble bees [6] and toads [7,8], preferentially direct help toward relatives, while tactics such as punishing/policing cheats have been well documented, for example, in queenless ants [9], honeybees [10] and humans [11,12].

Our understanding of how cooperators adapt to the presence of cheats has been greatly enhanced by studies of microbes, due to their suitability for carrying out real-time evolution experiments as well as identifying the genetic basis of behaviours. Maintaining high relatedness between producer and beneficiary is paramount for allowing cooperation to persist [13], so that directing the benefits of cooperation to close kin or clone mates can mitigate the negative influence of cheats on population growth [14]. Preferential interactions with close relative can be facilitated by a spatially structured environment [15], but even in the absence of spatial structure mechanisms such as 'green beard' genes [16,17] and antagonistic pleiotropy [18,19] facilitate directing the benefits of cooperation to kin.

Several studies have demonstrated real-time evolution of cheat resistance in cooperator populations. *Pseudomonas fluorescens* biofilms can be invaded by non-contributing cheats, compromising the integrity of the biofilm. Coevolution between these two phenotypes gives rise to increasingly efficient cheats and more resistant cooperators [20]. Moreover, cooperators have been found to evolve ways of opposing productivity of cheats who fail to contribute to dead-end stalk cells in *Myxococcus xanthus* [21], and *D. discoideum* [22]. However, the exact mechanism of cheat resistance in these cases is unclear.

While such examples involve direct physical association between producer and cooperative trait, it is less clear if and how adaptation may occur in an anonymous public good scenario, where there is usually no clear physical link between producer and product. Hence, directly targeting cheats for punishment/policing or directing benefits of cooperation toward kin is problematic. Moreover, simple point mutations often lead to the rapid production of cheats, making pleiotropy unlikely as an anti-cheating mechanism [23]. Several recent studies have suggested novel ways by which bacteria can fine-tune their cooperative output to ensure beneficiaries are highly related. For instance, quorum sensing can be used by bacteria to infer when they are surrounded by clone mates, allowing them to tune their investment into cooperative traits depending on the genotype of surrounding cells [24,25]. Similarly, *Escherichia coli* plasmid donors can bias altruistic transfer of beneficial plasmids only to other cells that share the donation alleles [26]. However, this requires a degree of population structuring or an association between transfer and discrimination alleles.

Here, we investigate the real time evolutionary response of cooperators to cheats when cooperation is mediated by a public good that is individually costly and carries a group-level benefit: the production of iron-scavenging siderophores by *Pseudomonas aeruginosa*. Cheats evolve rapidly in this system, avoiding the cost of siderophore production while still retaining the correct receptor for uptake of the siderophore-iron complex [1, 27]. **Two** recent studies coevolved siderophore producers and cheats together, and reported the evolution of reduced siderophore production in producer populations [28,29]. However, in these studies either the fitness consequences of altered siderophore production were not assessed [28] or the experimental design did not impose selection for cooperation, so that cheating, rather than resistance to cheating, was selected for [29]. As such, these studies did not determine whether or not cooeprators can adapt to cheats. Here, we evolve *P. aeruginosa* in the presence or absence of cheats, under conditions where there is selection for cooperation: patches within a metapopulation are mixed and single co-operator clones from this mixture used to inoculate new patches. Mixing patches means that genotypes from the most productive patches, i.e. in which cooperators are less exploited by cheats, are overrepresented in subsequent generations, while those from less productive patches are underrepresented. Inoculating new patches with single clones resulted in high relatedness, and hence stronger selection for cooperation [30].

Materials and methods

Strains and growth media

The *P. aeruginosa* strain PAO1 was used as the siderophore-producing wildtype. A gentamicin-resistant PAO1 (PAO1^{R}) and gentamicin-resistant PAO1 with a *lacZ* reporter gene insertion (PAO1 ^{R}*lacZ)* were engineered by integrating a gentamicin

resistance cassette (Tn7-gm) and a *lacZ* gene (with a gentamicin resistance cassette; Tn7-gm-*lacZ*), respectively at the *att*::Tn7 locus in *P. aeruginosa* PAO1 [31]. PAO1*ΔpvdDΔpchEF* is a gentamicin-susceptible isogenic mutant strain of PAO1 with genes encoding both primary and secondary siderophores, pyoverdine and pyochelin knocked out [32]. Experiments were carried out in Kings Medium B (KB) [33]: (10 g glycerol, 20 g proteose peptone no. 3, 1.5 g $K_2HPO_4.3H_2O$, 1.5 g $MgSO_4.7H_2O$ per litre). Where stated, KB medium was made iron-limited by the addition of freshly made filter-sterilised 100μg/ml human apotransferrin and 20mM $NaHCO_3$ to KB medium immediately before use. Since siderophore production is repressed when there is an excess of Fe^{2+} [34]; iron-limitation ensures that siderophores are essential for growth and stimulates their production. Gentamicin was used at a concentration of 30 μ g/ml and 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) at 90 μ g/ml. Bacteria were grown at 37ºC shaken at 180rpm unless stated otherwise.

Costs and benefits of siderophore production

We firstly confirmed that siderophore production carries a cost, and is exploitable by non-producing cheats in our experimental context: 6ml iron-limited shaken KB medium. We established six PAO1 populations (cooperator), six PAO1*ΔpvdDΔpchEF* populations (cheat) and six populations in 1:1 co-culture, quantifying relative fitness of cheats after 24h. Proportions of bacteria were inoculated so that the density of bacteria added to each microcosm was ~ 10^7 CFU's ml^{-1} (colony forming units). Bacterial densities were assessed by plating appropriate dilutions on KB agar after 24h growth.

Evolution Experiment

We evolved cooperators ($PAO1^R$) in the presence and absence of a high frequency (90%) of siderophore-negative cheats (PAO1*ΔpvdDΔpchEF*) (figure A1). This high frequency of cheats ensures there is selection for cooperators to adapt to mitigate the adverse effects of cheats on population productivity. Our design comprised of 6 replicate populations for each treatment (+/- cheats), with each population consisting of 6 'patches'. Patches were initiated with a single cooperator colony; 10^7 CFU's ml^{-1} for the control treatment and 10^6 CFU's ml^{-1} for the 90% cheat treatment. $9x10^6$ CFU's ml^{-1} PAO1*ΔpvdDΔpchEF* were also added to the 90% cheat treatment so that the final inoculated bacterial density was also 10^7 CFU's ml^{-1}. After 24h growth, 100μl was combined from each patch and plated on KB agar + gentamicin (facilitating cheat removal). Single colonies were then selected at random to inoculate new patches, and cheats were re-added to the appropriate treatment, using the same number of CFU's as before. This design (global competition and high relatedness) selected for subpopulations with high productivity, since genotypes from the most productive subpopulations were overrepresented in the mixture (see [18]) This process of enforcing global competition was repeated 18 times (~190 generations), but cultures were allowed to grow for 96 hours (rather than 24h) between the enforcement of the final 4 rounds of global competition, transferring 1% of the cells to fresh media every 24h. This was to accelerate evolutionary change which we speculated was being constrained by daily bottlenecking of cultures.

Quantifying fitness of evolved populations: competition experiments

Fitness of each of our 12 evolved populations was assessed relative to their ancestor in both selective environments: in the presence and absence of cheats. Six replicates were established per population in 6ml Fe-limited medium, totalling 144 fitness

175 assays. Half of the tubes were inoculated with ~5x10^5 CFU's ml^{-1} ancestral
176 PAO1^R*lacZ* (gentamicin-resistant and *lacZ* insertion strain), ~5x10^5 CFU's ml^{-1} of the
177 appropriate evolved population and ~9x10^6 CFU's ml^{-1} of PAO1*ΔpvdDΔpchEF*, so
178 that cheat strains represented a high proportion (~90%) of the total bacterial density in
179 each microcosm, and the total inoculated density was 10^7 CFU's ml^{-1}. A further 72
180 microcosms (cheat-free competitions) were inoculated with ~5x10^6 CFU's ml^{-1}
181 ancestral PAO1^R*lacZ* and ~5x10^6 CFU's ml^{-1} of the appropriate evolved population,
182 so the total inoculum was 10^7 CFU's ml^{-1}. Microcosms were grown for 24h, after
183 which densities were assessed by plating liquid cultures on KB agar supplemented
184 with 30μg/ml gentamicin and 90μg/ml Xgal, and counting viable colonies.
185 Gentamicin facilitated the removal of cheats at the counting stage, which otherwise
186 would have dominated the plates and resulted in very low cooperator counts. Evolved
187 and ancestral cooperator strains were distinguished by a dark blue appearance of the
188 ancestral strain on Xgal-supplemented agar. Finally, the neutrality of the *lacZ*
189 insertion in the ancestral strain under these growth conditions was confirmed by
190 competing PAO1 ^{R}*lacZ* with PAO1^R at 1:1 in Fe-limited KB media.
191
192 *Measuring public goods production*
193 After ~190 generations, each replicate was diluted and cultured on KB agar to
194 measure: i) colony morphotypic variation; ii) *per capita* siderophore production; iii)
195 production of the most costly and efficient iron-siderophore, pyoverdine [35-37]; iv)
196 the toxin pyocyanin, which can generate soluble iron.
197
198 Thirty randomly selected colonies from each population were statically grown in
199 200μl iron-limited KB medium (siderophore-stimulating conditions). *Per capita* total

siderophore production was quantified by combining 50µl from each of the 30 single colony cultures, centrifuging to pellet cells and performing a 50% Chrome azurol S (CAS) assay on the supernatant, measuring A_{630} of cultures as well as the cell-free supernatant (reference culture) [38,39]. A measure of iron chelator activity relative to the reference culture in each population was given by $[1-(A_{pop}/A_{ref})]$, standardised by the optical density (A_{600}) of the relevant culture. *Per capita* pyoverdine was quantified for each of our 30 isolated colonies per population using a pyoverdine-specific emission assay [40]. Briefly, fluorescence of each culture was measured at 460nm following excitation at 400nm, using a Biotek Synergy 2 Spectrophotometer. Optical density (OD) was measured at 600nm, and the ratio RFU/OD was employed as a quantitative measure of *per capita* pyoverdine production. Finally, evolved populations were analysed for production of the toxin pyocyanin, which can promote soluble ferrous iron [41]. Briefly, all evolved populations were plated on gentamicin-supplemented agar to remove cheats. Wildtype cells (with gentamicin resistance) were washed from plates using 6ml KB broth, and grown overnight in 30ml glass tubes. After 24h, cells were centrifuged, and A_{691} was measured for each population, standardised by A_{600}.. Ancestral wildtype, cheat and uninoculated KB broth were included in all of our assays as controls.

Quantifying evolved population productivity

To investigate whether any adaptation to cheats in treatment populations sacrificed population productivity, the relative growth rate of each evolved population was assessed by growing each evolved population for 24h in Fe-limited KB media. Final densities were quantified by plating liquid cultures on KB agar and counting viable colonies.

225

226 *Addition of exogenous pyocyanin*

227 We investigated whether pyocyanin could rescue the poor fitness of siderophore
228 cheats in an iron-limited environment. We tested the effect of 10μM, 30μM and
229 50μM pyocyanin on the growth of both PAO1 (cooperator) and siderophore negative
230 cheat PAO1*ΔpvdDΔpchEF,* relative to a pyocyanin-free control. 10^7 CFU's ml^{-1} of
231 cooperator or cheat was inoculated into 6ml iron-limited pyocyanin-supplemented KB
232 and grown for 24h. Final densities were assessed by plating on KB agar.

233

234 *Resequencing methods and bioinformatic analysis.*

235 The Wizard® Genomic DNA Purification kit (Promega) was used to isolated genomic
236 DNA from overnight cultures, according to manufacter's instructions. The quality of
237 the isolated gDNA was assessed using Nanodrop (Thermo Scientific). Four smooth
238 and four novel morphotypes from end point cheat-adapted populations were selected
239 for sequencing. TruSeq PCR-free genomic libraries were prepared at the Centre for
240 Genomic Research, University of Liverpool and 2x 250bp paired-end reads generated
241 on an Illumina MiSeq platform. See S1 for further details on sequence data
242 preparation.

243

244 *Statistical analyses*

245 All data were analysed using R version 2.15.1 [42]. We determined population
246 Malthusian growth rate (m) as ln(final density/start density) [43]. Relative fitness of
247 strain x compared with strain y (W(x)) was calculated in co-culture as m(strain
248 x)/m(strain y), and in monoculture as m(strain x)/mean(m(strain y)). When W(x) = 1,
249 fitness of strain x = strain y. Following an F-test to compare variances, and a Shapiro-

250 Wilk normality test, we used Student's t –tests and Wilcoxon rank-sum tests to
251 compare *m* values in monoculture, or *W* values between treatments. To assess whether
252 (W(*x*)) was significantly different from 1, we used 1-sample t-tests or Wilcoxon
253 signed-rank tests.
254
255 1 and 2-sample t-tests were used to compare *per capita* total siderophore production
256 between evolved populations and between evolved populations and the ancestor
257 (using mean ancestral siderophore production as the alternative value in 1-sample
258 tests), and a Kolmogorov-Smirnov test for non-parametric data with unequal
259 variances was employed to compare the frequency of a novel morphotype in control
260 and treatment populations. A 1-sample t-test, and Wilcoxon signed-rank was used to
261 assess whether evolved pyoverdine production in control and treatment populations
262 differed significantly from that of the ancestor. To compare per capita pyoverdine
263 between evolved populations, we used a linear mixed effects revised (LMER) model,
264 assigning condition (treatment/control) as a fixed factor and population as a random
265 factor, controlling for the presence of 30 datapoints for each of 12 populations. To
266 determine fitness of evolved populations relative to ancestor in the presence/absence
267 of cheats, a LMER model was employed to account for non-independent datapoints (6
268 replicates per population), assigning 'population' as a random factor and both
269 condition (treatment/control) and cheats (present/absent) as fixed explanatory factors
270 (including interaction). A general linear model (GLM) was used to investigate
271 whether pyocyanin production is affected by evolution condition (control/treatment
272 lines). The relationship between the proportion of novel morphotypes and *per capita*
273 production of pyoverdine and pyocyanin was examined using separate generalised
274 linear models with a quasibinomial error structure. Finally, the effect of exogenous

pyocyanin on growth rate was calculated as the change in growth relative to the control at each pyocyanin concentration using selection coefficient (r): m(strain x) - mean(m(strain y)). Using a GLM, the effect of pyocyanin concentration (continuous numeric variable) and strain identity (cooperator or cheat) on promoting growth was investigated (including the interaction). To ensure the effect of adding 10μM pyocyanin was accounted for, the control treatment relative selection coefficient was included and standardized to zero.

Results

Costs and benefits of siderophore production

As with previous studies in iron-limited minimal media [30,44], monocultures of cooperators exhibited a higher growth rate compared with cheat monocultures (Wilcoxon rank-sum test: W=34, $p<0.05$), however this effect was reversed in 1:1 co-culture, where cheats had a growth rate advantage over cooperators (1-sample t-test of relative fitness against 1: $t_5=2.74$, $p<0.05$, figure A2). Thus, while siderophore production carries a group-level fitness benefit, is individually costly in this context.

Quantifying fitness against ancestor and productivity of evolved populations

To determine adaptation to cheats, we competed evolved lines against the ancestor in the presence and absence of 90% cheats: the same conditions as the selective environments. Populations evolved with cheats had a higher fitness than populations evolved in the absence of cheats, but only when cheats were present (LMER treatment x cheat interaction; $X^2_{1,5}=24.04$, $p<0.0001$, figure 2).

Given that our experimental design also selected for high within-population yields, we measured the mean growth rate of evolved populations as monocultures under the selective (iron-limited) conditions, finding no difference in mean growth rate between control and treatment lines (Student's t-test, t_{10}= 0.03, p=0.98, figure A3). Finally, we verified that the use of an ancestor possessing a *lacZ* genetic marker in this experiment did not alter relative fitness: in direct 1:1 competition with non-*lacZ* ancestor, W(*lacZ*) did not differ from 1 (1-sample t-test (alt=1), t_5=-1.57, p= *0.18*).

Public goods production following evolution

After ~ 190 generations of growth, populations grown in the presence of cheats exhibited reduced per capita total siderophore production compared with ancestor (1-sample t-test (alt=0.7), t_5=3.41, p<0.05, figure 1A) and cheat-free control populations (Student's t-test, t_{10}=2.77, p<0.05, figure 1A). Testing for pyoverdine specifically, treatment populations showed decreased pyoverdine output compared with ancestor (1-sided Wilcoxon signed-rank test, alternative = 7876.51, V=4, p<0.0001, figure 1B) and control populations (LMER: $X^2_{1,3}$=9.79, p <0.01, figure 1B). Control populations did not differ from ancestral total per capita siderophore production (1-sample t-test (alt=0.7) t_5=*0.7*, p = 0.51, figure 1A), but per capita pyoverdine output was reduced over the course of the experiment (1-sample t-test, alternative = 7876.51, t_{179}=18.63, p<0.0001, figure 1B).

Novel morphotypes

We recorded the appearance of a novel morphotype with a slightly raised surface and reduced surface area in evolved populations (Figure A4, Table A1). After ~190 generations, not only was the frequency of this morphotype significantly higher in our

treatment than control populations (Kolmogorov- Smirnov test, D=0.83, p<0.05), it went to fixation in 3 out of 6 treatment populations (Table A1). The proportion of evolved novel morphotypes within a population was positively correlated with pyocyanin production (GLM, $F_{1,10}$=21.22, p<0.001, Table A1), and negatively associated with pyoverdine production (GLM, $F_{1,10}$=35.64, p<0.001, Table A1). Notably, population level pyocyanin production was only elevated relative to control when the proportion of novel morphotypes in a population reached fixation, and this variation between populations meant that we did not detect significant differences in total pyocyanin between populations (GLM, $F_{1,10}$=3.03, p=0.11, Table A1). We confirmed that the novel morphotype showed elevated pyocyanin and reduced pyoverdine production relative to ancestral-like colonies, by performing individual assays (replicated thrice) on the eight colonies subsequently selected for sequencing (see below) (pyoverdine: LMER, $X^2_{1,4}$=3.94, p<0.05; pyocyanin: LMER, $X^2_{1,4}$=26.51, p<0.0001).

To identify the mutation(s) that might confer this wrinkly pyocyanin-overproducing phenotype, the genomes of the ancestral, four smooth (isolated from treatment populations T1, T6 and T3) and four novel morphotypes (isolated from treatment populations T2, T4, T5 and T6) were sequenced and subjected to comparative genomic analysis. We observed deletions of 38.33Kb (on average) in the novel phenotypes compare to the smooth and ancestral strains (Fig A5, Table A2, A3). Bioinformatic analysis revealed that the deleted genomic fragments contain 19 genes common to all novel morphotypes, including *lasR* and *rsaL* genes encoding a regulator and a repressor of quorum sensing (QS) regulated factors, respectively (Table A3). These genes were not mutated in any sequenced smooth colonies. Among

the four smooth colonies, three in-frame deletions were observed in three different colonies: in *fha1* (2/3) and *ftsY* (1/3) - neither of which are associated with iron-acquisition.

Addition of exogenous pyocyanin

The addition of exogenous pyocyanin was beneficial for both siderophore-producing and non-producing strains; however, the effect of increasing pyocyanin concentration on growth rate was greatest for non-producing strains (GLM, strain identity x pyocyanin concentration interaction, $F_{1, 44}=12.02$, $p=0.001$, figure 3).

Discussion

Here, we investigated whether populations of the bacterium *P. aeruginosa* under selection for high productivity were capable of adapting to the presence of public goods cheats, where public goods in this case are iron-scavenging siderophores. We found that after ~190 generations, cheat-adapted populations manifested greater fitness in the presence of cheats compared with control populations, while displaying no apparent growth rate cost when grown in the absence of cheats. While evolved populations had significantly reduced pyoverdine production relative to the ancestor (adaptation to growth conditions), cheat-adapted populations further reduced their production of pyoverdine compared with control populations. Novel morphotypes appeared in 5/6 cheat-adapted populations, characterised by deletions in *lasR* and *rsaL* genes. The morphotype showed elevated pyocyanin and reduced siderophore production, and its presence resulted in population-level reductions in pyoverdine, while fixation of the morphotype in 3/6 cheat-adapted populations resulted in increased pyocyanin production. Taken together, these data suggest that one way

374 cooperators may have adapted at least in part to the presence of siderophore cheats is
375 by down-regulating siderophore production while up-regulating an alternative means
376 to obtain iron (pyocyanin).
377
378 Our work has some parallels with recent work by Kümmerli *et al* [29] whereby
379 coevolution between *P. aeruginosa* siderophore cooperators and cheats drove reduced
380 pyoverdine output by cooperators and blockage of the costly pvdS signalling pathway
381 by coevolving cheats. However, the evolution of reduced pyoverdine in this study and
382 that observed by Kummerli *et al* is likely to have been driven by different selection
383 pressures. Notably, Kummerli *et al* performed experimental coevolution by
384 transferring 1% of each culture to new media daily. This design facilitates local
385 competition and low relatedness, which reduces pyoverdine production *per se,*
386 because cheating is favoured [30]. Conversely, the metapopulation design established
387 in our study ensures that any reduction in cooperative output is a direct consequence
388 of adapting to resist the impact of cheats rather than selection for cheating. In line
389 with this, Harrison [28] found that coevolving *P. aeruginosa* cooperators and cheats
390 in metapopulations that impose selection for cooperation reduced pyoverdine
391 production. However, in this case consequences of lowered pyoverdine for cooperator
392 fitness were not determined, and the coevolutionary design made it difficult to
393 disentangle the effects of altered relatedness and cheating [45].
394
395 We have speculated that reductions in pyoverdine and elevated pyocyanin in the novel
396 morphotype may have contributed to the observed adaptation to siderophore cheats in
397 some populations. Reduced pyoverdine production will presumably mitigate some of
398 the fitness costs imposed by pyoverdine cheats [37]. The resulting reduction in iron

399 acquisition may be compensated for by up-regulation of pyocyanin, whose canonical
400 function is a toxin [46]. Pyocyanin is a potent reducing agent that converts insoluble
401 ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron [41], which can diffuse into cells via cell-surface
402 porins (eliminating the requirement of a siderophore-specific receptor). Accordingly,
403 we found that the addition of exogenous pyocyanin had a stronger effect in enhancing
404 growth of siderophore-negative cheats compared with cooperators, suggesting it can
405 compensate for lack of siderophore production. Moreover, recent studies investigating
406 adaptation of *P. aeruginosa* to the antimicrobial gallium nitrate show that cells
407 become resistant by downregulating pyoverdine (which acts in this case as a gallium
408 transporter) and upregulating pyocyanin [47,48]. However, while populations with
409 high frequencies of novel morphotypes may have benefitted from this increase in
410 pyocyanin, the remaining populations are likely to have evolved alternative, unknown
411 strategies to cope with reduced pyoverdine production. One possibility is that prudent
412 regulation of cooperative traits can impede the spread of cheats, by only cooperating
413 when the costs of doing so are minimal. For example, the diffusible *P. aeruginosa*
414 carbon-rich rhamnolipid is expressed only when growth is limited by another nutrient
415 source [49]. However, in our experiment the costs of cooperating were consistently
416 high, based on our finding that cheats invaded cooperators when competed at 1:1.
417
418 The novel morphotype characterised by reduced pyoverdine and increased pyocyanin
419 production, carried deletions in *lasR* and *rsaL* genes. LasR and RsaL are two
420 transcriptional regulators that positively and negatively regulate the expression of QS
421 regulated virulence factors, respectively [50-52]. Pyoverdine production is under
422 positive control of the Las system and its inactivation has been reported to reduce the
423 production of this siderophore [53]. LasR also regulates pyocyanin production,

however the pyocyanin biosynthetic operon *phzA-G1* is under direct repression of RsaL and it has been shown that cells that lack *rsaL* overexpress pyocyanin [54]. It therefore seems likely that these deletions play a role in the observed phenotypic changes in the novel morphotype, although we can't rule out that other gene deletions in this novel morphotype may have contributed to changes in siderophore and pyocyanin production, as well as adaptation more generally.

Our finding that that the growth rate of siderophore cheats in iron-limited media can be rescued by the addition of exogenous pyocyanin suggests that like siderophores, pyocyanin may also act as a public good. This is further supported by studies in animal models demonstrating reduced growth and virulence of pyocyanin negative mutants compared with wildtype [55,56] but that mutant growth is enhanced by the presence of wildtype producers in mixed infections, or the addition of exogenous pyocyanin [56]. Note that in this case, pyocyanin is probably not linked to iron scavenging, as non-producers had intact siderophores, and pyocyanin has a range of additional *in vivo* activities such as apoptosis of neutrophils that could enhance growth. This then begs the question: why weren't pyocyanin overproducers in our evolved cheat-adapted populations exploited by individuals making less pyocyanin? The most likely explanation is that pyocyanin over-production imposes a small metabolic cost (relative to siderophores), at least under these experimental conditions; hence pyocyanin non-producers would have little, if any, fitness advantage. However, our results do not rule out the possibility that pyocyanin producers could ultimately be exploited by the evolution of pyocyanin cheats in this or other environments.

449 One counterintuitive result was the loss of fitness through time in evolved
450 populations: competing ancestral against evolved cooperator populations in the
451 presence and absence of cheats demonstrated that evolved control populations were
452 consistently outcompeted by the ancestor, while evolved treatment populations
453 managed to negate this only in the presence of cheats. The inherent disadvantage of
454 evolved populations could not be attributed to differences in growth between
455 PAO1^{R}*lacZ* and PAO1^{R}, and was potentially a consequence of population
456 bottlenecking resulting from transfer of single clones, which may have resulted in the
457 fixation of deleterious mutations [57].
458
459 It is always debatable whether *in vitro* results are relevant to the real world. While
460 siderophore mutants are present in natural populations [58,59] and can have a
461 selective advantage when rare [15] it is unclear if a) they act as cheats in this context
462 and b) if selection has acted in ways that mitigate their exploitation, as observed here.
463 Pyocyanin over-producing genotypes are associated with exacerbated cystic fibrosis
464 infections [60] and while it can be speculated that these phenotypes represent an
465 alternative mechanism of iron-acquisition, these correlational data can of course be
466 open to different interpretations. Nonetheless, the evolutionary impact of altered
467 social interactions between microbes should be carefully considered in all cases,
468 particularly in light of the development of novel therapeutics aimed at disrupting
469 microbial social interactions [56, 69]. Given that pyocyanin directly harms host cells,
470 kills competitors, and results in more virulent infections [54, 64, 68], investigation
471 into whether pyocyanin contributes significantly as an iron-uptake mechanism in
472 natural populations is warranted.
473

Data, code and materials

The datasets supporting this article have been uploaded as part of the supplementary material.

Competing Interests

The authors declare no conflict of interest

Authors Contributions

SOB carried out experimental work and statistical analysis, as well as designing the study and drafting the manuscript. SP performed genome sequencing and SP and AL interpreted genome sequences. MC and AB conceived the study, and AB also coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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Figure Captions

Figure 1: Total siderophore **(A)** and pyoverdine **(B)** production by evolved control populations (Ctrl.), treatment populations (Trt.; evolved in the presence of 90% cheats) and the ancestral clonal PAO1 (Anc.). After ~190 generations, treatment populations exhibited reduced per capita total siderophore production (1-sample t-test

(alt=0.70672), t_5=3.4056, p <0.05) and pyoverdine production (1-sided Wilcoxon signed-rank test, alternative = 7876.512, V=4, p<0.0001), relative to the ancestor. For **A,** data are means of 6 evolved populations for each treatment ± SEM, and the single population of ancestral PAO1. For **A,** data are means of pyoverdine production for 30 colonies for each evolved population (6 evolved populations each for control and treatment condition), and the single ancestral PAO1.

Figure 2: Relative fitness of evolved populations in a 1:1 co-culture with ancestral wildtype PAO1, in both the presence and absence of cheats, in iron-limited KB media. Evolved populations were generally less fit than their ancestor, with the exception of treatment populations when competed under the same conditions as which they had evolved in (in the presence of 90% cheats). (LMER treatment x cheat interaction: $X^2_{1,5}$=24.044, p<0.0001). Data are means of 6 replicates per each of 12 evolved populations ± SEM.

Figure 3: Effect of the addition of exogenous pyocyanin to PAO1 (cooperator; circles and dashed line) and PAO1*ΔpvdDΔpchEF* (cheat; triangles and solid line) populations. Selection coefficient is calculated relative to a control cooperator or cheat population to which no pyocyanin was added. The effect increasing pyocyanin has on relative fitness (r) is greatest in cheat populations (GLM, strain identity x pyocyanin concentration, $F_{1,44}$ =12.018, p=0.001). Fitness (r) relative to control population (no pyocyanin added) was calculated as m(strain x) – mean(m(strain y)). Lines are plotted based on predictions from minimal GLM model. Data are means of 6 replicates per treatment ± SEM.