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Co-evolutionary dynamics between public good producers and cheats in the bacterium *Pseudomonas aeruginosa*

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Abstract

The production of beneficial public goods is common in the microbial world, and so is cheating – the exploitation of public goods by nonproducing mutants. Here, we examine co-evolutionary dynamics between cooperators and cheats and ask whether cooperators can evolve strategies to reduce the burden of exploitation, and whether cheats in turn can improve their exploitation abilities. We evolved cooperators of the bacterium *Pseudomonas aeruginosa*, producing the shareable iron-scavenging siderophore pyoverdine, together with cheats, defective in pyoverdine production but proficient in uptake. We found that cooperators managed to co-exist with cheats in 56% of all replicates over approximately 150 generations of experimental evolution. Growth and competition assays revealed that co-existence was fostered by a combination of general adaptions to the media and specific adaptions to the co-evolving opponent. Phenotypic screening and whole-genome resequencing of evolved clones confirmed this pattern, and suggest that cooperators became less exploitable by cheats because they significantly reduced their pyoverdine investment. Cheats, meanwhile, improved exploitation efficiency through mutations blocking the costly pyoverdine-signalling pathway. Moreover, cooperators and cheats evolved reduced motility, a pattern that likely represents adaptation to laboratory conditions, but at the same time also affects social interactions by reducing strain mixing and pyoverdine sharing. Overall, we observed parallel evolution, where co-existence of cooperators and cheats was enabled by a combination of adaptations to the abiotic and social environment and their interactions.

Introduction

Bacteria frequently cooperate by forming multicellular fruiting bodies and biofilms, and by secreting shareable metabolites to digest food, scavenge essential metals and attack competitors (West *et al.*, 2007; Nadell *et al.*, 2009; Velicer & Vos, 2009; Strassmann & Queller, 2011). These cooperative traits are typically beneficial for the community, but can also be exploited by cheating mutants that stop contributing to costly cooperation, while still capitalizing on the cooperative acts performed by others (West *et al.*, 2006). This raises the question of how cooperation can be maintained given the pervasive risk of cheat exploitation. Previous studies have identified a number of ecological and social factors, including resource availability (Brockhurst *et al.*, 2008; Kümmerli *et al.*, 2009c; Xavier *et al.*, 2011), limited dispersal (Griffin *et al.*, 2004; Gilbert *et al.*, 2007; MacLean & Brandon, 2008; Kümmerli *et al.*, 2009b) and cell density (Greig & Travisano, 2004; Ross-Gillespie *et al.*, 2009), which can tip the balance in favour of

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cooperation. In contrast to these extrinsic drivers of cooperation, relatively little is known on whether cooperators and cheats can directly adapt to one another, and co-exist under conditions that would normally favour cheating (Zhang *et al.*, 2009; Khare *et al.*, 2009; Hollis, 2012; Levin *et al.*, 2015).

Here, we investigate whether cooperators and cheats co-evolve, eventually engaging in antagonistic co-evolution, as it is typically observed in host–parasite interactions (Decaestecker *et al.*, 2007; Schulte *et al.*, 2010; Gomez & Buckling, 2011; Morran *et al.*, 2011; Thrall *et al.*, 2012). In cooperative systems, cheats can behave analogous to parasites, such that similar evolutionary dynamics might arise. For instance, cooperators could adapt to the presence of cheats by: (i) an obligate reduction in cooperation; (ii) a facultative reduction in cooperation when encountering cheats; or (iii) making the cooperative trait less exploitable (Khare *et al.*, 2009; Manhes & Velicer, 2011; Ghoul *et al.*, 2014a; Levin *et al.*, 2015). Cheats, meanwhile, could counter-adapt by: (iv) evading any form of cheat recognition required for (ii); or (v) improving their access to cooperators and their beneficial acts. In addition, it is also possible that cooperators and cheats adapt to abiotic conditions, which might allow social traits to hitchhike along with beneficial nonsocial mutations (Morgan *et al.*, 2012; Waite & Shou, 2012; Asfahl *et al.*, 2015).

We examined these possibilities by investigating the evolutionary response of repeatedly interacting cooperator and cheat strains to one another and to the abiotic environment in the bacterium *Pseudomonas aeruginosa*. Specifically, we co-evolved a producer of the shareable iron-scavenging molecule pyoverdine (i.e. cooperator) with a mutant that is defective in pyoverdine production but proficient in uptake (i.e. cheat) in iron-depleted media, where pyoverdine is important for growth. Pyoverdine is produced and secreted by *P. aeruginosa* in response to iron limitation and is used to scavenge insoluble or host-bound iron from the environment (Schalk & Guillon, 2013). Numerous experiments have shown that secreted pyoverdine molecules can be shared with other cells in the community, including nonproducing 'cheats' (Griffin *et al.*, 2004; Harrison *et al.*, 2006; Jiricny *et al.*, 2010; Dumas & Kümmerli, 2012).

We co-evolved populations of cooperators and cheats for 25 rounds of growth (approximately 150 generations), and followed cooperator frequency, population growth and pyoverdine production levels over time. Following evolution, we analysed evolved clones from different time points using a combination of fitness assays, phenotypic assays and whole-genome resequencing. This allowed us to examine, both at the proximate and ultimate levels, the ability of cooperators and cheats to adapt to each other, and to disentangle co-evolutionary dynamics from adaptations to the abiotic environment.

Materials and methods

Strains

We used *P. aeruginosa* PAO1 (ATCC 15692) as the wild-type cooperator strain, and the pyoverdine knockout mutant PAO1Δ*pvdD* (lacking the gene for the pyoverdine synthetase PvdD) as the cheating strain (Ghysels *et al.*, 2004). To be able to distinguish cooperators from cheats during co-evolution, we used variants of these strains constitutively expressing the green fluorescent protein GFP (PAO1-*gfp* and PAO1Δ*pvdD-gfp*, chromosomal insertion: *att*Tn7::Ptac-*gfp*).

Experimental co-evolution

Prior to experimental evolution, we grew strains overnight in 10 mL lysogeny broth (LB). We then standardized cultures for cell density [optical density (OD) at 600 nm], and prepared two 1 : 1 strain mixes (mix1: PAO1-*gfp* vs. PAO1Δ*pvdD*; mix2: PAO1 vs. PAO1Δ*pvdD-gfp*, to control for GFP-marker effects). We started experimental evolution by adding 10^6 cells into 1.5 mL iron-depleted CAA medium in 16-fold replication (eight replicates for each mix) on a 24-well plate. Iron-depleted CAA medium contained 5 g L^{-1} casamino acids, 1.18 g L^{-1} $K_2HPO_4*3H_2O$, 0.25 g L^{-1} $MgSO_4*7H_2O$, 100 μg mL^{-1} human apo-transferrin, 20 mM $NaHCO_3$ and 25 mM HEPES buffer (all from Sigma-Aldrich, Buchs, Switzerland). Apo-transferrin is a powerful natural iron chelator, which we used to bind ferric iron in the CAA media, thereby preventing siderophore-independent iron uptake. After a 24-h growth period under static conditions at 37 °C, during which approximately six generations occur (Dumas & Kümmerli, 2012), we carried out the following experimental steps: (i) we measured OD (at 600 nm) and pyoverdine production (fluorescence at excitation|emission = 400|460 nm) using a multimode plate reader (Tecan Infinite M200 PRO; Tecan Group Ltd, Männedorf, Switzerland) (Kümmerli *et al.*, 2009c); (ii) we diluted culture aliquots to appropriate levels in 0.8% NaCl, and plated 30 μL onto LB agar containing 100 μM $FeCl_3$ to assess strain frequency ($FeCl_3$ was supplemented to suppress residual pyoverdine production, which can interfere with the GFP-signal); (iii) mixed 200 μL of the culture with 100 μL LB and 100 μL glycerol for long-term storage at −80 °C; and (iv) transferred 15 μL of the culture to fresh medium (corresponding to a 100-fold dilution) to initiate the next round of growth. We counted colony-forming units (CFU) on LB agar plates following a 48-h incubation period (24 h at 37 °C followed by 24 h at room temperature). We differentiated GFP-tagged from nontagged colonies using a Dark Reader Transilluminator (Clare Chemical Research, Dolores, CO, USA). The above procedure was repeated for 25 consecutive rounds of growth, resulting in approximately 150 generations of experimental co-evolution.

Time-shift competition assays

To test whether evolved cooperators became better at coping with cheats, and/or evolved cheats improved their ability to exploit cooperators, we competed ancestral and evolved clones from various time points against each other in all possible pairwise combinations. Specifically, we isolated a total of 896 clones (on average 8.8 ± 1.8 and 9.5 ± 1.2 clones of cooperator and cheat origin, respectively) from the 5th, 10th, 15th, 20th and 25th round of growth. This was possible for 11 of the 16 replicates, where the two strains co-existed for most of the experimental period (i.e. three replicates until 20th round; eight replicates until 25th round). In the other five replicates, cheats completely displaced cooperators, which concomitantly resulted in population collapse and extinction. For each round–replicate combination, we grew the isolated clones individually in 96-well plates in CAA without apo-transferrin, conditions under which all clones reached similar OD after 24 h (mean OD ± SE = 0.460 ± 0.007). We then mixed equal volumes (50 μL) of all cooperator or cheat clones originating from the same round–replicate combination in Eppendorf tubes. With these mixes, we set up 1 : 1 competitions between cooperators and cheats in all possible pairwise combinations, such that cooperators competed against cheats from their past, presence and future. Following a 24-h competition period in iron-depleted CAA medium under static conditions at 37 °C, we plated appropriately diluted fractions of the competition cultures onto LB plates. Following a 48-h incubation period, we counted the GFP-tagged vs. non-tagged strains as described above. Using CFU data, we calculated the relative fitness of cheats as $v = [x_2(1 - x_1)]/[x_1(1 - x_2)]$, where x_1 and x_2 are the starting and final frequency of cheats in the population, respectively. x_1 was based on the ratios of the OD of the starting cultures and was typically close to 0.5. We log-transformed all fitness values prior to analysis to obtain normally distributed residuals.

Growth and competition assays to test for adaptation to the media and/or the social environment

We first tested for adaptation to the laboratory (abiotic) environment by growing monocultures of all 896 clones used for the time-shift competition assay in the medium they have evolved in (i.e. iron-depleted CAA). If clones significantly improve their growth performance in this assay, then this would indicate that strains have adapted to abiotic conditions. We measured OD at 600 nm of all clones after a 24-h static growth assay at 37 °C. OD after 24 h is a good proxy for fitness under the strongly growth-limiting conditions imposed in our experiment because strains grow slowly, linearly over time and do not reach carrying capacity within 24 h (see Fig. S1 and Kümmerli *et al.*, 2009c). Thus, any beneficial mutation shortening the lag phase and/or increasing growth rate will result in higher OD. We scaled ODs of evolved strains relative to the ancestral wild type.

In addition to these clonal assays, we competed evolved cooperator and cheat clones (from the end of the co-evolution) directly against their respective ancestors in mixed culture (using the same protocol as described above). For this assay, it is important to note that competitive ability can still be influenced by both abiotic and social adaptations. Thus, if evolved cooperators were found to outcompete their ancestors, this could result from adaptation to media, as well as from the opportunity to exploit any surplus pyoverdine produced by ancestral strains. To disentangle social from abiotic fitness effects, we performed competition assays in iron-deplete media, where both social and abiotic adaptations should play a role, and iron-replete media, where pyoverdine is not needed and therefore only abiotic adaptations should matter.

Another possibility to implement control treatments would have been to evolve cooperator and cheat strains in isolation from each other (Brockhurst & Koskella, 2013). However, this is difficult with our system because cheat monocultures grow poorly, such that these cultures would have likely gone extinct during the serial passages of the evolution regime (Fig. S2, and also see Fiegna & Velicer 2003). Furthermore, *de novo* cheats arise quickly in cooperator monocultures (Harrison *et al.*, 2008; Dumas & Kümmerli, 2012), such that this treatment would have turned from a control into a co-evolution treatment.

Sequencing and SNP analysis

We sequenced the entire genome of 90 cooperator and 105 cheat clones from the end of the experimental co-evolution (R25 for 8 replicates, and R20 for three replicates) using Illumina HiSeq 2000. The evolved 195 clones were arranged in 30 pools as listed in Table S1. We pooled the cheat clones originating from the same replicate (11 pools in total). The cooperator clones were also pooled per replicate but also based on phenotypes. Specifically, we identified clones with significantly altered pyoverdine production levels in some replicates. For these replicates, we assembled the clones with and without changed pyoverdine production levels in separate pools (19 pools in total). Finally, we also sequenced our ancestral wild-type strain PAO1.

We extracted genomic DNA using the Wizard Genomic DNA purification kit (Promega, Dübendorf, Switzerland). Extracted DNA was sent off for commercial library preparation and sequencing with Illumina HiSeq 2000 using paired-end 50-bp reads (paired-end and single reads for 20 and 11 pools, respectively; GATC Biotech, Constance, Germany). Data analysis

was performed in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich. In a first step, we mapped the contigs of our wild-type strain onto the reference genome of PAO1 (Stover *et al.*, 2000), PAO1-UW (www.pseudomonas.com). The average sequence coverage was high (222), and the consensus length of our resequenced wild-type strain was 99.985% of the reference genome (6 264 404 bp). Reference and resequenced wild-type strain differed in 25 SNPs (10 nonsynonymous, 5 synonymous and 10 intergenic SNPs, see Table S2). Next, we mapped the contigs of our evolved clones onto the resequenced genome of our ancestral wild-type strain. Whenever a single clone was sequenced, we considered a putative mutation as a SNP if its frequency was $\geq$ 80%. In cases where clones were pooled for sequencing, we used the following threshold frequencies for putative mutations to be considered as SNPs (for two clones: frequency $\geq$ 25%; for three clones: frequency $\geq$ 16.7%; for four clones: frequency $\geq$ 12.5%; for five clones: frequency $\geq$ 10%; for six clones: frequency $\geq$ 8.3%; for seven clones: frequency $\geq$ 7.1%; for eight clones: frequency $\geq$ 6.3%; for nine clones: frequency $\geq$ 5.6%; for 10 clones: frequency $\geq$ 5%). We discarded putative mutations with coverages < 15. Using these criteria, we identified 62 nonsynonymous SNPs in coding DNA sequences, and 19 SNPs in intergenic regions (Table S1).

Swarming assays

Because we identified SNPs in genes involved in flagella synthesis and chemotaxis (Table S1, for cooperators in 8 of 11 replicates; for cheats in 9 of 11 replicates), we tested whether clones isolated from pools with such SNPs showed altered motility phenotypes. For this analysis, we focused on a subset of samples (five cooperator and cheat pools each). For each of the 31 cooperator and 45 cheat clones from these pools, we quantified swarming motility on 0.4% LB agar in 3-fold replication. Specifically, we grew clones overnight in 10 mL LB medium at 200 r.p.m. at 37 °C. We then washed cells in PBS (phosphate buffer saline), adjusted OD to 1, and added 2 μL of the cell solution to the centre of a Petri dish containing 20 mL 0.4% LB agar. Dishes were incubated statically for 24 h at 37 °C. Following incubation, the dishes were placed individually on 1-mm-grid paper and photographed. We analysed the pictures with GIMP 2.8 (GNU Image Manipulation Program, freely available from http://www.gimp.org), by quantifying the number of pixels covered by the swarming colony. The 1-mm grid was used to calculate the surface s (in cm^2) covered by the swarm. We then calculated the relative swarming of evolved strains as $r_s = (s_{evolved} - s_{PAO1\Delta rhlA})/(s_{PAO1} - s_{PAO1\Delta rhlA})$, where $s_{evolved}$, s_{PAO1}, $s_{PAO1\Delta rhlA}$ are the swarm surfaces of the evolved, ancestral wild type and a swarming-knockout strain (PAO1Δ*rhlA*, which lacks the gene for the production of rhamnolipids, biosurfactants essential for swarming), respectively. Evolved strains were considered swarming impaired if $r_s < 0.75$.

Simulating the effect of motility impairment on pyoverdine sharing

SNPs in motility genes most likely represent adaptation to laboratory conditions (Ritchings *et al.*, 1995; Velicer *et al.*, 1998). However, motility deficiency potentially feeds back on social interactions between cooperators and cheats by limiting strain mixing, which in turn can reduce the cheats' access to pyoverdine (see Kümmerli *et al.*, 2009b). Because such a feedback is difficult to examine experimentally, we used a simulation platform specifically designed to study social interactions in microbes (Dobay *et al.*, 2014). This platform provides a two-dimensional continuous landscape, in which public goods-producing cooperators and nonproducing cheats are seeded. Simulations typically start with one cooperator and one cheat cell, and stop when populations reach the carrying capacity K (number of individuals). Cooperator cells produce public good molecules at a specified rate p (molecules per second) and with specific properties (diffusion coefficient D_{pg} and durability δ). Public good production (per molecule) comes at a cost c, whereas public good uptake generates a benefit b. Cooperator and cheats can themselves diffuse (i.e. are motile), whereby D_{co} and D_{ch} describe their respective diffusion coefficients. The fitness functions of cooperators and cheats are described in detail in Dobay *et al.* (2014), and are basically the sum of the intrinsic growth rate μ (not influenced by public goods), the benefits generated by public good uptake and the cost of public goods production accruing to cooperators.

As a baseline for our simulations, we chose parameter settings that closely match our experimental system – a liquid environment where cell and public good diffusion is relatively high, and where public goods are important for growth ($p = 1$, $D_{pg} = 5\ \mu m^2\ s^{-1}$, $\delta = 510$ s, $c = 0.001$, $b = 0.01$, $D_{co} = D_{ch} = 5\ \mu m^2\ s^{-1}$, $\mu = 1$, $K = 500$). With these settings, cheats significantly outcompete cooperators, demonstrating the fact that cheats can free ride on the public goods produced by cooperators. To investigate how motility impairment (as observed in our experiment) feeds back on the relative success of cooperators and cheats, we simulated situations in which cooperators and/or cheats acquire mutations reducing their cell diffusion coefficient from 5 to 0.5 $\mu m^2\ s^{-1}$. We simulated two scenarios, one where motility reduction is neutral (constant $\mu = 1$), and one where motility reduction is beneficial, increasing μ by 0.2%. For each parameter combination, we ran 500 independent simulations.

Statistical analysis

We used linear models (LM) and linear mixed models (LMM) for statistical analyses. We tested whether the frequency of cheats, absolute and relative fitness, and levels of pyoverdine production changed over evolutionary time. Furthermore, we tested for correlations between genotypes (SNPs) and phenotypes (growth, pyoverdine production, and motility). As repeated measures were taken from the same replicates over time, we included replicate ID as a random factor into our model. We also accounted for the fact that clones from the same replicate are not independent by averaging across clones prior to analysis. All statistical analyses were carried out with R 3.1.1 (R Development Core Team, 2015).

Results

Evolutionary dynamics of strain frequency, growth and pyoverdine production

We found that cheats significantly increased in frequency over evolutionary time (LMM: $t_{332} = 10.96$, $P < 0.0001$), with average cheat frequency raising rapidly during the first part of the experiment, but then levelling off (Fig. 1a). There was also a significant GFP-marker effect (LMM: $t_{14} = 3.93$, $P = 0.0015$), but only at the beginning of the experiment (significant interaction between round and marker: LMM: $t_{332} = 6.32$, $P < 0.0001$). When looking at individual replicates, we observed that cheats became fixed in 7 of 16 replicates (fixation events occurred in rounds 13, 21, 23 and 25), whereas cooperators co-existed with cheats across the entire duration of the experiment (mean cheater frequency $\pm$ SE = 0.71 $\pm$ 0.04 in the remaining nine replicates).

During experimental evolution, both population growth (LMM: $t_{382} = -10.16$, $P < 0.0001$, Fig. 1b) and population-level pyoverdine production (LMM: $t_{382} = -15.41$, $P < 0.0001$, Fig. 1c) significantly decreased over time. When comparing across replicates, we found that end point values of cheat frequency were significantly negatively correlated with evolved population growth (Pearson's product–moment correlation: $r = -0.894$, d.f. = 14, $P < 0.0001$), and evolved pyoverdine production levels ($r = -0.764$, d.f. = 14, $P = 0.0006$) (Fig. S2), showing that the spreading of cheats typically drives population growth and pyoverdine production towards zero.

Time-shift competition assays suggest co-evolutionary dynamics

Among the replicates where cooperators and cheats co-existed for at least 20 experimental rounds, we found that cooperators from later evolutionary time points performed significantly better in competition with cheats than cooperators from earlier time points (Fig. 2a–f; LM: $t_{319} = -4.67$, $P < 0.0001$). Similarly, cheats from later evolutionary time points became increasingly better at outcompeting cooperators (Fig. 2a–f), as indicated by the consistent significant positive relationships between cheat round of origin and relative cheat fitness (LM: $t_{319} = 5.21$, $P < 0.0001$). In contemporary competitions (i.e. competitions where cooperators and cheats originate from the same round), cheats significantly outcompeted cooperators ($t_{58} = 2.06$, $P = 0.044$), suggesting that cooperators remained vulnerable to exploitation at any given moment in time.

Abiotic and social adaptations influence evolutionary dynamics

The competitive advantage of evolved strains in the time-shift experiment could not only have arisen due

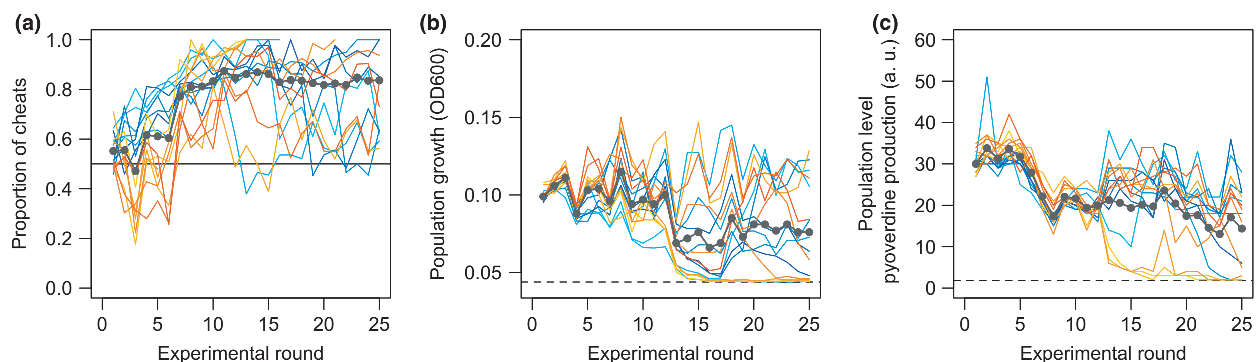

Fig. 1 Evolutionary dynamics of cheat frequency (a), population growth (b) and pyoverdine production levels (c) in mixed populations of cooperative pyoverdine producers and non-pyoverdine-producing cheats of the bacterium *Pseudomonas aeruginosa*. Experimental evolution occurred across 25 rounds of growth (approximately 150 bacterial generations). Cheat frequency first increased and then levelled off. Population growth and pyoverdine production significantly dropped over time. Grey lines depict averages across 16 replicates. Blue and orange lines show replicates in which the cooperator or cheat strain was labelled with a neutral GFP marker, respectively.

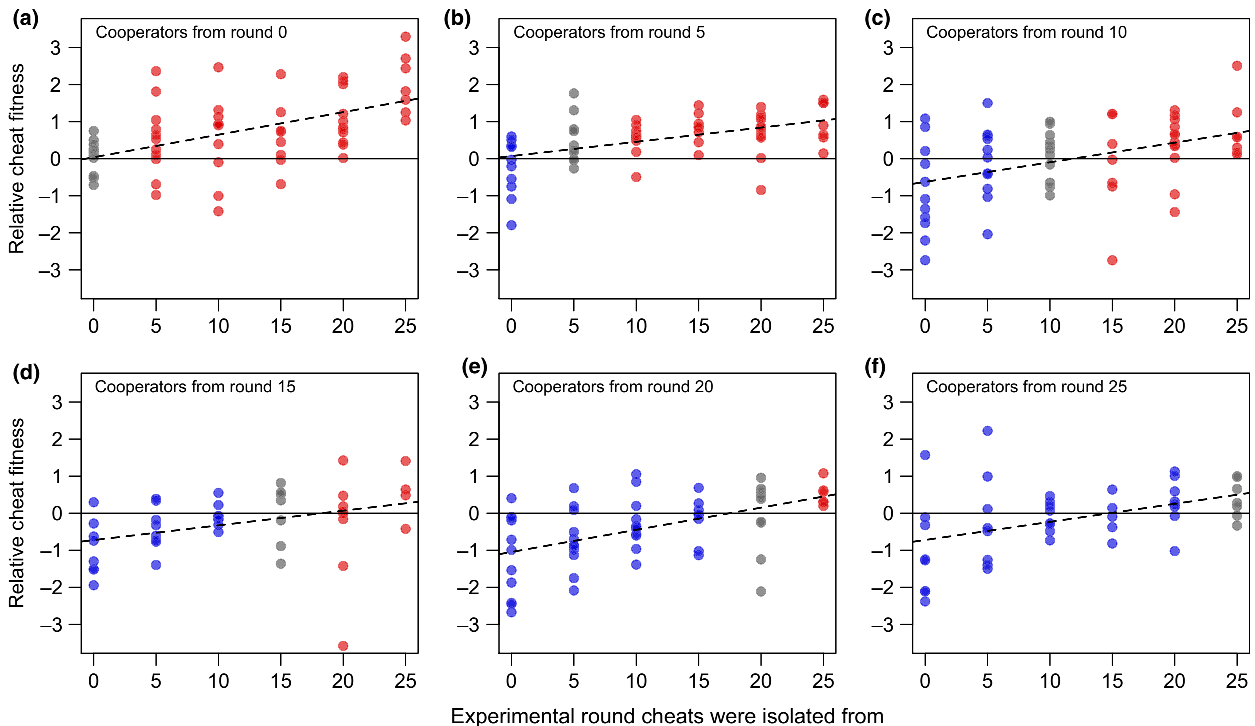

Fig. 2 Relative cheat fitness as a function of the evolutionary origin of cheats (along the *x*-axis) and cooperators (from a to f). Origin of both cheats and cooperators significantly affected cheat fitness, as indicated by the dashed trend lines, and the overall reduction in cheat fitness when moving from (a) to (f). These patterns suggest that cooperators from later evolutionary time points were less exploitable by cheats and that cheats from later evolutionary time points became increasingly better at outcompeting cooperators. Blue, red and grey circles indicate combinations where cooperators competed against cheats from their past, future and present, respectively. Circles above or below the zero-line indicate that cheats won or lost the competition, respectively.

to social interactions, but also through adaptation to the abiotic environment. Our monoculture experiments, in which we grew evolved cooperator and cheat clones apart suggests that media adaptation occurred: cooperator and cheat clones slightly but significantly improved their growth over evolutionary time (Fig. 3a, LMM for cooperators: $t_{48} = 2.81$, $P = 0.007$; for cheats: $t_{48} = 2.17$, $P = 0.035$). However, another insight gained from these monoculture experiments was that the cooperators' pyoverdine production levels significantly dropped over evolutionary time (Fig. 3b, LMM for cooperators: $t_{48} = -6.43$, $P < 0.0001$), indicating that selection also acted on the social trait of interest. In support of the hypothesis that both social and abiotic adaptations drove the evolutionary dynamics observed in Fig. 2, we found that, in competitions between evolved and ancestral cooperators, the relative fitness of evolved cooperators was significantly higher under iron-deplete conditions, where both social and abiotic adaptations played a role, than under iron-replete conditions, where only abiotic adaptations mattered (Fig. 3c, paired *t*-test: $t_9 = 6.93$, $P < 0.0001$). Competitions between ancestral and evolved cheats, meanwhile, were less informative because these cultures hardly managed to grow. But also here, we found evidence for media adaption since the evolved cheats grew slightly but significantly better than their ancestor (mean number of doublings in 24 h: evolved vs. ancestral cheats = 2.53 ± 0.40 vs. 0.41 ± 0.11, paired *t*-test: $t_8 = 6.36$, $P = 0.0002$).

Sequencing analyses reveal mutations in social and nonsocial genes

To better understand the genetic basis of the observed evolutionary dynamics, we sequenced genomes of 195 evolved clones from the end of the experiment. We found that nonsynonymous SNPs repeatedly arose in three functionally different regions of the genome (Table S1), which correspond to: (i) sequences coding and regulating the iron starvation sigma factor PvdS (13 putative independent mutation events); (ii) sequences coding for various genes involved in flagella synthesis and regulation (16 putative independent mutation events); and (iii) sequences coding for genes involved in chemotaxis (11 putative independent mutation events). Intriguingly, mutations in all three regions were found both in the cooperator and the cheat

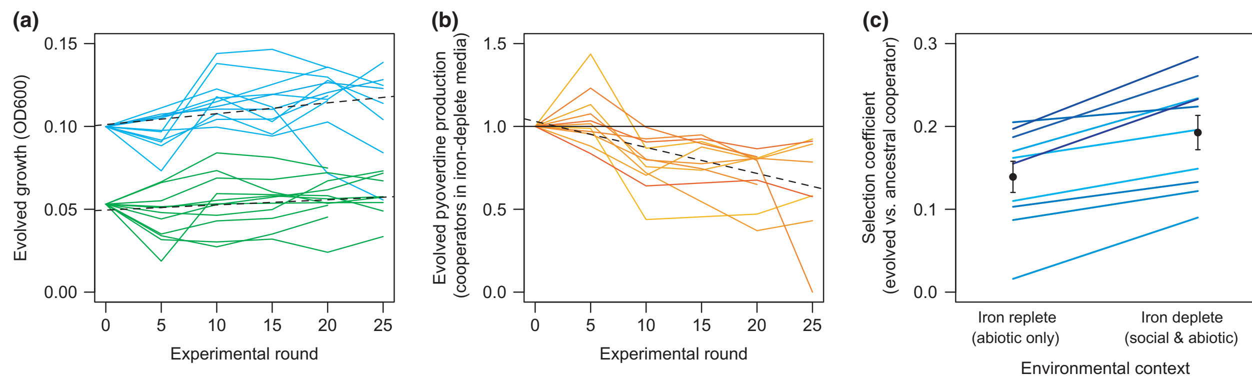

Fig. 3 Experiments testing for media vs. social adaptations. (a) Growth of evolved clones in monocultures slightly but significantly increased over evolutionary time both for cooperators (blue lines) and cheats (green lines) indicating adaptation to the media. (b) Analysis of the clonal pyoverdine production profiles revealed a significant drop over evolutionary time, suggesting that also the social trait of interest was under selection. (c) Evolved clones consistently outcompeted their ancestors both in iron-replete and iron-deplete environments (selection coefficient > 0). In support of the hypothesis that both social and abiotic adaptations drove the evolutionary dynamics observed in our system, we found that the selection coefficient of evolved cooperators was significantly higher under iron-deplete conditions, where both social and abiotic adaptations were important, than under iron-replete conditions, where only abiotic adaptations matter. All values in (a) and (b) are scaled relative to the ancestral wild type and represent means across clones from the same replicate. Colour shadings depict the different replicates and dashed lines represent significant trend lines.

background (number of events in cooperator vs. cheat background for *pvdS* region: 8/5; for flagella genes: 9/7; for chemotaxis genes: 4/7).

Phenotypes and fitness associated with SNPs in *pvdS* region

There was a strong association between the reduced levels of pyoverdine production among cooperator clones and the presence of mutations in the *pvdS* region (Table S1). Specifically, clones with *pvdS* mutations showed significantly lower pyoverdine production levels than clones without *pvdS* mutations (pyoverdine production level relative to the ancestral wild type, mean ± SE, for clones with *pvdS* mutations: 0.25 ± 0.06; without *pvdS* mutations: 0.99 ± 0.04; LM: $F_{1,16} = 95.29$, $P < 0.0001$; Fig. 4). Moreover, the presence/absence of *pvdS* mutations correlated with clonal fitness. Cooperator clones without *pvdS* mutations grew significantly better than clones with *pvdS* mutations (LM: $F_{1,16} = 9.73$, $P = 0.007$), and also better than the ancestral wild type ($t_9 = 4.07$, $P = 0.0028$, Fig. 4). Evolved clones with *pvdS* mutations varied a lot in their growth performance (in some cases growth decreased dramatically), but overall, there was no significant drop in growth relative to the ancestral wild type ($t_6 = -1.42$, $P = 0.199$, Fig. 4).

Cheat clones with *pvdS* SNPs were phenotypically indistinguishable from the ancestral cheat and typically did not fix (i.e. the sequenced pools consisted of a mix of clones with and without mutations in *pvdS*, Table S1). For those reasons, we could not link mutations to fitness. However, there was one replicate (No. 8, Table S1) in which all sequenced cheat clones had a mutation in the *pvdS* region. These clones showed growth almost identical to their ancestor (mean growth ± SE: 0.99 ± 0.03, $t_9 = 0.29$, $P = 0.78$).

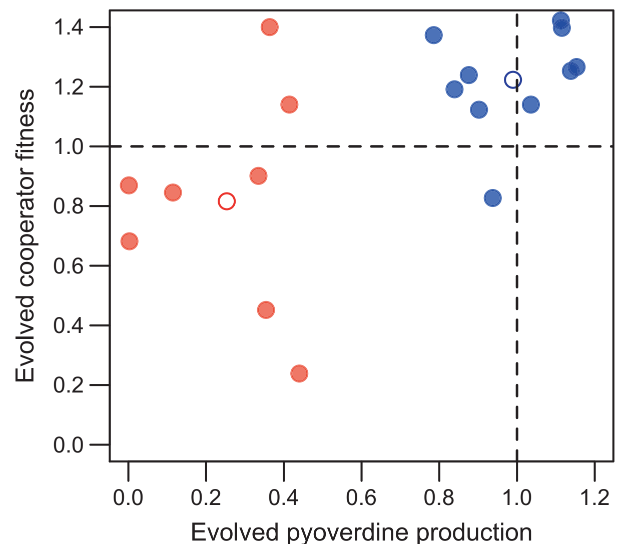

Fig. 4 Relationship between SNPs in the iron starvation sigma factor PvdS, pyoverdine production and fitness (growth yield in monoculture) of evolved cooperator clones. Evolved clones with SNPs in the *pvdS* region (red circles) produced significantly less pyoverdine and had significantly lower fitness than evolved clones without SNPs in the *pvdS* region (blue circles). All values are scaled relative to the ancestral wild type (dashed lines). Closed circles depict means across clones from the same replicate and mutation event, while open circles represent averages across replicates.

Phenotypes and fitness associated with SNPs in flagella and chemotaxis genes

Across the subset of sequenced pools analysed, we consistently found that the presence of SNPs in flagella and chemotaxis genes went along with the presence of clones showing significantly reduced swarming (mean relative swarming compared to the ancestral wild type $r_s \pm$ SE: 0.374 ± 0.054). Evolved clones with motility impairment grew marginally significantly better than the ancestral wild type ($t_{10} = 2.06$, $P = 0.066$, Fig. 5), but not significantly different from the evolved strains without motility impairment ($F_{1,15} = 0.40$, $P = 0.537$).

Simulations reveal that motility impairment promotes cooperation

Our experiments were carried out in static liquid medium, where flagellated wild-type bacteria can easily mix and siderophores can readily diffuse. Under such conditions, cheats typically outcompete cooperators (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009b). However, how do the competitive abilities change when strains become motility impaired as observed in our co-evolution experiment? To address this question, we used computer simulations where we could manipulate bacterial motility in a fully controlled *in silico* environment (see Materials and Methods for details). When simulating ancestral conditions, where bacteria are highly motile, we indeed found that cooperators lose in competition with cheats (cooperator frequency drops from 0.5 to 0.256). Next, we implemented our empirical observation that strains became motility impaired. When assuming that motility impairment confers no fitness benefit, our simulations reveal that cooperators increase in frequency, relative to the standard conditions described above, no matter whether motility impairment occurred in cooperators (frequency increase from 0.256 to 0.319), cheats (from 0.256 to 0.264) or both strains (from 0.256 to 0.370). Finally, we simulated the case where motility impairment confers a small fitness benefit and where both strains became motility impaired (as observed in our experiment). Also under these conditions, we found that cooperators increased in relative frequency (from 0.256 to 0.399). These results indicate that motility impairment benefits cooperators more than cheats because it leads to more local sharing of public goods among cooperators.

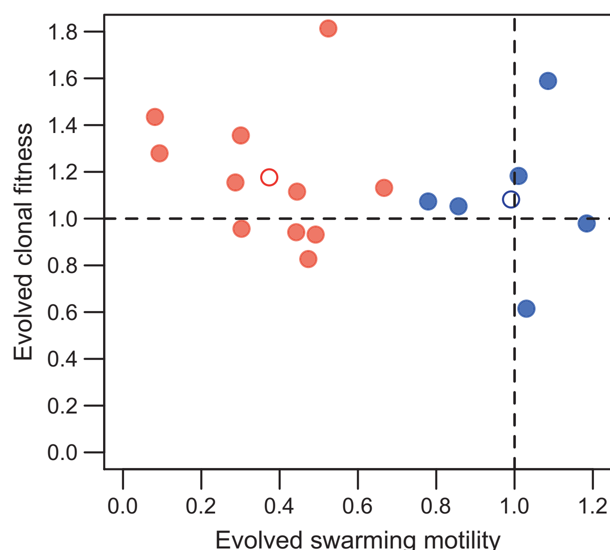

Fig. 5 Relationship between swarming motility and fitness (growth yield in monoculture) of evolved clones isolated at the end of the evolution experiment. Swarming assays revealed that a large number of evolved clones were motility impaired (red circles, $r_s < 0.75$). Evolved clones with motility impairment grew marginally significantly better than the ancestral wild type, but not different from evolved strains without motility impairment (blue circles). All values are scaled relative to the ancestral wild type (dashed lines). Closed circles depict means across clones from the same replicate and mutation event, while open circles represent averages across replicates.

Discussion

Our co-evolution study with *P. aeruginosa* revealed that pyoverdine-producing cooperators could co-exist with pyoverdine-exploiting cheats across 150 bacterial generations in the majority (56%) of replicates. Our phenotypic and sequencing analyses suggest that co-existence was fostered by cooperators and cheats adapting to both one another and the abiotic environment. Adaptations to the co-evolving opponent included cooperators significantly down-regulating their pyoverdine production, which lowered the overall level of cooperation, and cheats blocking costly pyoverdine signalling, which is triggered upon pyoverdine uptake. Adaptations to the abiotic environment included a reduction in motility, which could potentially feed back on social interactions between cooperators and cheats by limiting strain mixing and pyoverdine sharing.

We found that an obligate reduction in cooperation by 75%, owing to point mutations in the gene and promoter region of the iron starvation sigma factor PvdS arose repeatedly in many replicates. This illustrates how the presence of cheats can favour public good producers to reduce their level of cooperation (Dumas & Kümmerli, 2012; Ghoul *et al.*, 2014a). However, these partial-pyoverdine producers hardly ever fixed, such that populations generally consisted of a mixture of ancestral full-pyoverdine producers, evolved partial-pyoverdine producers and nonproducers. The evolved partial-pyoverdine producers could therefore take on a double role in the population: they can be regarded as cooperators in competition with the nonproducer, but potentially act as cheats in competition with the ancestral wild-type pyoverdine producer (Ghoul *et al.*,

2014b). Further work is required to determine whether this diversity of pyoverdine strategies can remain stable in the long run, by a mechanism such as frequency-dependent selection (Ross-Gillespie *et al.*, 2007; MacLean & Gudelj, 2006; Ross-Gillespie *et al.*, 2015).

We further observed that point mutations in the iron starvation sigma factor region also repeatedly occurred in the cheat background. The spreading of these mutants can be explained by the fact that our ancestral cheat (defective for the pyoverdine synthetase PvdD) is still receptive to pyoverdine-mediated signalling (Lamont *et al.*, 2002), whereby the uptake of pyoverdine triggers the expression of genes involved in the synthesis of pyoverdine precursors. Signalling is silent when cheats grow in monoculture, but becomes activated in co-culture with pyoverdine producers (Tiburzi *et al.*, 2008), consequently leading to substantial costs associated with cheating. These costs can be eliminated by mutations in the iron starvation sigma factor (Tiburzi *et al.*, 2008). Thus, the spreading of these mutations can be understood as a direct response to the presence of cooperators.

What role do adaptations to the abiotic environment, such as the observed reduction in motility, play in the co-evolutionary dynamics between cooperators and cheats? Previous work suggested that cooperative traits could hitchhike along with mutations that provide benefits outside the social context (Morgan *et al.*, 2012; Waite & Shou, 2012; Asfahl *et al.*, 2015). The reasoning is that in cooperative systems cheats must invade from rare, and therefore, any beneficial (nonsocial) mutation is more likely to occur among cooperators because they are more numerous. This could finally result in selective sweeps, during which the beneficial mutation fixes, the cooperative trait hitchhikes along, and cheats are purged (Waite & Shou, 2012). We did not find support for this scenario. For one thing, we started with equal proportions of cooperators and cheats such that hitchhiking could work in favour of both strains depending on in which background the beneficial (nonsocial) mutation arises first. However, even when taking this into account, we found no clear evidence for hitchhiking based on selective sweeps. One reason for the absence of large-scale hitchhiking might be that motility impairment seems to confer only small fitness benefits (Fig. 5). Moreover, simulations suggest that motility impairment feeds back on the social interaction between cooperators and cheats as it limits strain mixing, which in turn can lead to more local sharing of public goods among cooperators, hampering the selective advantage of cheats. Taken together, our findings indicate that adaptations to the abiotic environment can interact with social components of the environment, and do not necessarily favour hitchhiking.

Do the observed adaptations indicate that cooperators and cheats engage in antagonistic co-evolution, characterized by cooperators becoming resistant against cheating and cheats improving exploitation abilities? The answer is 'no', as we did not observe cooperators to evolve mechanisms preventing public good exploitation while maintaining high levels of cooperation. Nonetheless, in our study the evolution of reduced pyoverdine production and the feedback of motility impairment on social interactions significantly helped to reduce the burden of cheating and allowed cooperators to co-exist with cheats for much longer periods than would be expected based on the results from short-term invasion experiments (Griffin *et al.*, 2004; Ross-Gillespie *et al.*, 2007; Kümmerli *et al.*, 2009c). Furthermore, the mutations in the iron starvation sigma factor increased the relative fitness of cheats when grown with cooperators that produced pyoverdine. Thus, while we did not observe antagonistic co-evolution, we did observe both cooperators and cheats becoming better adapted to a social environment that includes the other type.

Would the observed response of cooperators, to reduce pyoverdine production and impair motility, help sustain some level of cooperation in the long run? Again, the answer is 'no'. First of all, we observed cheat fixation in 7 of 16 replicates. This is by itself not surprising because we chose experimental conditions that are highly unfavourable for cooperation, as we combined high costs of cooperation (induced by strong iron limitation) with low relatedness (thousands of cells were transferred and no population structure was implemented) (Griffin *et al.*, 2004; Kümmerli *et al.*, 2009a). Second, even in the remaining 9 replicates, where strains co-existed more stably, the long-term persistence of cooperation is not guaranteed, as exemplified by the evolutionary dynamics observed in replicate no. 11 (Table S1). In this replicate, a motility-reducing flagella mutation fixed early among cooperator clones, allowing the evolved cooperators to co-exist with cheats. Subsequently, two independent mutations in the iron starvation sigma factor occurred that resulted in the complete abolishment of pyoverdine production, such that the final population consisted of a mixture of ancestral and *de novo* evolved pyoverdine nonproducers. Although reflecting an extreme case, this example shows that a single point mutation suffices to turn a reasonably well-adapted cooperator into a *de novo* cheat. These considerations suggest that some form of population structure, generating significant levels of relatedness among interacting individuals (i.e. public goods are more likely shared among cooperators), might be required to stabilize cooperation (Griffin *et al.*, 2004; MacLean & Gudelj, 2006; Diggle *et al.*, 2007; Kümmerli *et al.*, 2009a; Rumbaugh *et al.*, 2012).

Finally, we can speculate about whether cheating resistance is possible in our system and what putative mechanisms could confer it. The evolution of cheating resistance combined with sustained high levels of cooperation was indeed observed in a study where only cooperators but not cheats were allowed to evolve

(L. A. Santorelli, R. Kümmerli, M. Toll-Riera, A. S. Griffin & S. A. West, unpublished data) While the underlying resistance mechanism remained unclear in this particular study, cheating resistance could principally evolve via a modified, more specific pyoverdine that is no longer accessible to cheats. This scenario has indeed previously been put forward to explain the existing pyoverdine diversity (Smith *et al.*, 2005; Lee *et al.*, 2012), and has also been suggested to drive diversification in bacterial quorum-sensing communication systems (Eldar, 2011). One reason for why diversification did not appear in our experiment might be that at least two mutations are required to change pyoverdine specificity – one that alters pyoverdine structure and one that adjusts receptor specificity accordingly. The odds for this to occur within our relatively short experimental period are conceivably low. Thus, the question of whether cooperator–cheat antagonism can drive diversification in social systems remains still open.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Continuous linear growth of ancestral cooperator (blue line) and cheat (red line) monocultures in iron-depleted CAA medium.

Figure S2 At the end of the evolution experiment, the evolved community level pyoverdine production (a) and population growth (b) were significantly negatively correlated with the proportion of cheats, showing that cheat accumulation first drives cooperation and then the entire population to extinction.

Table S1 Nonsynonymous and intergenic SNPs in evolved cooperator and cheat clones.

Table S2 SNPs of the PAO1 wild-type strain used in this study compared to the reference PAO1-UW (http://pseudomonas.com).

Data deposited at Dryad: doi: 10.5061/dryad.45kd1

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