Combinatorial quorum sensing in *Pseudomonas aeruginosa* allows for novel cheating strategies.

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Abstract

In the opportunistic pathogen *Pseudomonas aeruginosa*, quorum sensing (QS) is a social trait that is exploitable by non-cooperating cheats. Previously it has been shown that by linking QS to the production of both public and private goods, cheats can be prevented from invading populations of cooperators and this has been termed 'a metabolic incentive to cooperate'. We hypothesized *P. aeruginosa* could evolve novel cheating strategies to circumvent private goods metabolism by rewiring its combinatorial response to two QS signals (3O-C12-HSL and C4-HSL). We performed a selection experiment that cycled *P. aeruginosa* between public and private goods growth media and evolved an isolate which rewired its control of cooperative protease expression from a synergistic (AND-gate) response to dual signal input, to a 3O-C12-HSL only response. We show that this isolate circumvents metabolic incentives to cooperate and acts as a combinatorial signaling cheat, with a higher fitness in competition with its ancestor. Our results show three important principles; first, combinatorial QS allows for diverse social strategies to emerge, second, that restrictions levied by private goods are not sufficient to explain the maintenance of cooperation in natural populations and third that modifying combinatorial QS responses can have important physiological outcomes, including changes to antibiotic resistance.

Introduction

Social traits and sociality in bacteria have received extensive attention in recent years, from the exploitation of collective behaviors by cheats [9, 12, 21–24, 26, 29, 35, 40], to the implications for infection [7, 18, 26]. *Pseudomonas aeruginosa lasR* quorum sensing (QS) mutants have previously been shown to act as social cheats in environments where QS is required for growth. This is because cheater cells have a higher relative fitness than wildtype strains in mixed populations when QS is required for maximum fitness because they exploit QS-dependent exoproducts produced by wildtype cells [4, 10, 12, 22, 29]. QS *lasR* mutants are also frequently isolated from cystic fibrosis (CF) lungs [4, 32], and they readily arise during long-term selection experiments where the QS controlled production of public goods can be easily exploited [13, 29]. More recently, QS signals themselves have been shown to be exploitable by *lasI* signal cheats in specific environments [24].

Given the ease by which QS cheats can evolve and spread, solutions must exist to maintain QS in natural populations. Kin selection theory states that social behaviors can

be favored if the benefits preferentially promote the survival of individuals sharing those traits [16, 20]. Kin selection has been shown to be an important factor in maintaining cooperative behaviors, including QS, in microbes [4, 12, 13, 15, 17, 27]. Recently, Dandekar et al. demonstrated that by linking QS to both public and private goods (exoprotease production and adenosine metabolism respectively), lasR cheats can be prevented from enriching in co-culture with wildtype cells, as 'cheats' suffer a loss of direct fitness due to their failure to exploit private goods. This mechanism for restricting the spread of QS cheats was termed 'a metabolic incentive to cooperate' [9].

We tested whether bacterial cells can utilize novel and more complex forms of cheating to exploit loopholes in QS-dependent private goods metabolism, as suggested by our recent work on the functional roles of multi-signal QS systems [8]. P. aeruginosa has a complex QS regulatory architecture, featuring behavioral responses to multiple signal inputs. In previous work, we proposed that by using combinatorial (non-additive) responses to multiple signals with different environmental half-lives, individual cells are better able to resolve both social (density) and physical (flow rates/containment) dimensions of their environment. We specifically predicted that secreted proteins will be under synergistic or 'AND' gate control, to tailor expression to favorable high density and low mass transfer environments. Consistent with this prediction, we found that P. aeruginosa secreted proteins are controlled synergistically by the QS signal molecules N-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) [8].

Our earlier predictions were made under the assumption of clonal exploitation of a local environment, i.e., adaptation in the absence of cheats. However combinatorial signaling opens the door to novel social strategies, by tuning the extent of production and response to combinations of signals [8]. Tuning combinatorial responses can in principle decouple the regulation of traits that are individually advantageous (e.g., adenosine metabolism) from traits that are collectively beneficial (e.g., secretion of a costly digestive exo-enzyme). For example, if a cheat was capable of responding to just one signal to control a privately beneficial trait, then losing a response to a combination of QS signals could result in the cheat being able to do several things: (1) survive restrictions levied by private goods metabolism as the cell is still capable of activating metabolism by responding to the single signal; (2) exploit the production of public goods by cooperators by producing lower amounts of public goods, because cooperators responding to multiple signals will produce a greater amount than the cheat. Therefore, combinatorial cheats would potentially enrich at the expense of wildtype cells responding synergistically to multiple signals [8].

We performed a selection experiment explicitly designed to test whether cells can evolve to exploit synergistically-regulated public goods cooperation, given a metabolic incentive to cooperate. We cycled a P. aeruginosa double QS signal mutant (Δ*lasI/rhlI*) between growth in private and public goods media, with defined concentrations of synthetic signals. Having a double synthase mutant allowed us to control the signal concentration and test the combinatorial response to experimentally defined signal environments. Cycling the media allowed for a selection pressure on the private good metabolism to be maintained (in adenosine) while allowing the evolution of the public good-dependent responses (Bovine Serum Albumin, BSA). We show that (1) an isolate emerged that can circumvent metabolic incentives to cooperate; (2) this isolate acts as a cheat and has a higher fitness in competition with its ancestor and (3) disruption of combinatorial signaling was not directly linked to mutations in the known QS cascade of *P. aeruginosa*. Our findings highlight that combinatorial sensing allows for novel cheating strategies which mean that bacteria can exploit social behaviors in a number of different ways.

Materials and Methods

Bacterial strains and growth conditions. We used three strains in this study, and we specifically generated PAO-JG2 for the work. This strain is generated from PAO-JG1, a double QS signal synthase insertion mutant (ΔlasI/rhlI) [8]. We integrated a *lasB::luxCDABE* fusion constructed using the mini-*CTX::lux* system [6] into the chromosome of PAO-JG1 to create PAO-JG2. We also used PAO-JG1 as an unlabeled *lux* strain for competition assays. The other strain we used in this study is NCRi, a mutant of PAO-JG2, generated in an evolution selection experiment (see below). We routinely grew liquid cultures of strains at 37°C with shaking (200 rpm) in 5 ml of modified Quorum Sensing Medium (QSM) [10]. We monitored the growth of bacterial cultures by measuring the absorbance of light at a wavelength of 600 nm (optical density, OD_{600} using a spectrophotometer (Tecan 200i). We supplemented M9 agar plates with 0.4% adenosine as the sole carbon source [9].

Selection experiment. To test whether bacteria can avoid restrictions imposed by the metabolism of private goods by exploiting combinatorial signaling, we designed an experimental evolution approach (Fig. S1). Our experimental protocol (by design) prevented the de novo generation and spread of *lasR* mutants, but allowed for mutants that could exploit combinatorial signaling to evolve and avoid the levies imposed by QS-dependent private goods metabolism. Our selection experiment was performed in 6 replicate lines (each containing 4 microcosms) using PAO-JG2 as the ancestor strain, and we cycled evolving populations between QSM [10, 12] and an M9 agar media supplemented with adenosine as the sole carbon source (Figure S1). We grew PAO-JG2 for 48 h in QSM media at 37°C/200 rpm, and we supplemented the media with 1 μM of each C4-HSL and 3O-C12-HSL. We then plated the 4 microcosms of each replicate line onto adenosine M9 agar plates for 48 h. This plating restricts the passage of lasR mutants as they are unable to grow with adenosine as the sole carbon source. As we wished to study novel forms of cheating, we removed *lasR* mutants. We selected single colonies from the plates for each of the 4 new microcosms which we used to seed the next round of selection in QSM media. We ran the selection experiment 10 rounds (a single round being the cycle between growth in liquid QSM and on adenosine plates), which equates to $\approx$ 70 generations. After each round, we stored whole selected populations in QSM containing 25% glycerol -80°C. After 10 rounds of selection, we tested individual isolates for altered responses to QS signals (*lasB::lux* expression). We standardized *lasB::lux* measurements by dividing relative light units (RLU) by optical density (OD_{600}), resulting in per cell average *lasB* expression. We used peak level responses for analysis, and all peaks fell between 7-10 hours after initiation of growth. We randomly selected a single individual isolate from the final population and found that it showed altered *lasB::lux* expression compared to the ancestor when both signals were added in isolation or combination.

Competition experiments. We competed the ancestor (PAO-JG2), or our evolved isolate (NCRi), with an unlabeled version of the ancestor strain (PAO-JG1). We started cultures at equal densities (OD_{600} from each was measured then equally partitioned to a final OD_{600} of 0.05), in QSM media ± Signals (3 treatments, no signal, 3O-C12-HSL, and both C4-HSL and 3O-C12-HSL each at 0.5 μM) for 18 h. We then diluted cultures to 10^{-6} CFUs (ca) and we plated them out onto LB agar plates. We counted the total number of colonies expressing light (using a light amplifying camera) and compared this to the total number of colonies. We calculated the relative fitness of strains using the formula $w = p1(1-p0)/p0(1-p1)$ where $p0$ and $p1$ are the proportion of the strain we were testing fitness for in the population before and after incubation respectively [25].

Genomics and whole genome sequencing. We prepared genomic DNA from 14 h cultures of PAO-JG2 and the evolved NCRi isolate. We extracted DNA using the Sigma GenElute Bacterial Genomic DNA kit following the manufacturer's guidelines.

We performed multiplexed, 150 bp Paired-end sequencing on the Illumina HiSeq3000 platform to an average depth of 150x coverage. We performed de novo assemblies using Spades [5] annotated the assembled genomes using Prokka [31]. To investigate any large scale insertions or deletions between ancestral and evolved strain we performed comparative genomics by pairwise Blast analysis which we visualized using BRIG [3] and by generating a ProgressiveMauve genome alignment [11]. To determine the presence of SNPs in NCRi relative to the ancestral PAO-JG2 strain, we mapped the raw fastq data for NCRi against the contigs of the de novo assembled PAO-JG2 genome. Reads were first quality trimmed using Sickle, and them mapped to contigs using Bowtie2 and Samtools. We applied a SNP threshold of a minimum read depth of 10, minimum quality score of Q30, and a minimum allele frequency of 0.9 to call high fidelity SNPs. The raw sequence data for both strains has been deposited in the SRA under project accession number PRJNA437484.

Complementation with RhlR. We prepared competent P. aeruginosa cells by taking a 1% (v/v) inoculum from an overnight P. aeruginosa culture, adding to 50 ml of sterile LB medium and growing at 37°C/200 rpm to reach an OD_{600} of 0.4-0.5. We harvested cells by centrifugation at 5,000 rpm for 10 min at 4°C, and we then washed the cells three times in sterile ice-cold 300 mM (w/v) sucrose solution. We re-suspended cells in 200 μl of the ice-cold sucrose solution and then incubated for 30 minutes on ice. We performed electroporation in 0.2 cm electroporation cuvettes (Flowgen) containing 40 μl of competent cells and 2 μl of purified pUCP18::*rhlR*. We delivered an electroporation pulse of 1.6 kV using a BioRad Gene Pulsar connected to a BioRad pulse controller (BioRad Laboratories, Watford, UK). We then added a 1ml aliquot of LB broth to the cells and incubated for 1 h at 37°C/200 rpm before plating 100 μl onto selective LB agar plates containing carbenicillin (300 μg-$^{\text{ml}}$). We incubated plates overnight at 37°C and selected isolates containing plasmids the next day.

Chloramphenicol resistance. We determined MIC level by serial dilution in chloramphenicol from an initial range of 0 μgtextsuperscript-ml to a final concentration of 1600 μg-$^{\text{ml}}$. We grew bacteria overnight in 5 ml of LB with constant shaking at 37°C. After growth we washed the cells twice in PBS and resuspended in 200 μl antibiotic treatment in a 96 well plate at an OD_{600} of 0.05. We incubated the plates for 18 h at 37°C and MIC was determined as any well which did not increase in OD_{600} greater than 0.1.

Statistical analysis. We performed all statistical analyses using R (v3.4.2). We examined population selection lines by either an ANOVA, then applying posthoc Tukey HSD tests. When the data were not normally distributed, we used the Kruskal-Wallis tests within the car package for R. For MIC comparison a Welch two sample t test was performed.

Results

Combinatorial signaling determines P. aeruginosa fitness and QS-dependent gene expression in a QS-dependent environment. We first tested the impact of combinatorial signaling on strain fitness in an environment where QS is required for maximal growth (QSM) [10]. We used a PAO1Δ*lasI/rhlI* mutant strain (PAO-JG2) that makes no AHL signals, but which contains a *lasB::luxCDABE* chromosomal reporter fusion for monitoring QS-dependent gene expression. We measured the changes in *lasB* expression and PAO-JG2 growth in QSM with no added signals; C4-HSL or 3O-C12-HSL added in isolation; or a combination of both signals. We found using OD_{600} and relative light production, that both the growth of PAO-JG2 (Figure 1A) and lasB expression (Figure 1B) in QSM shows a synergistic ('AND-gate') combinatorial response. i.e., the response to dual signal inputs was greater than the additive combination of responses to single signal inputs alone ($X^2 = 8.3$ $p = 0.003$ and $X^2 = 6.8$ $p = 0.009$ respectively).

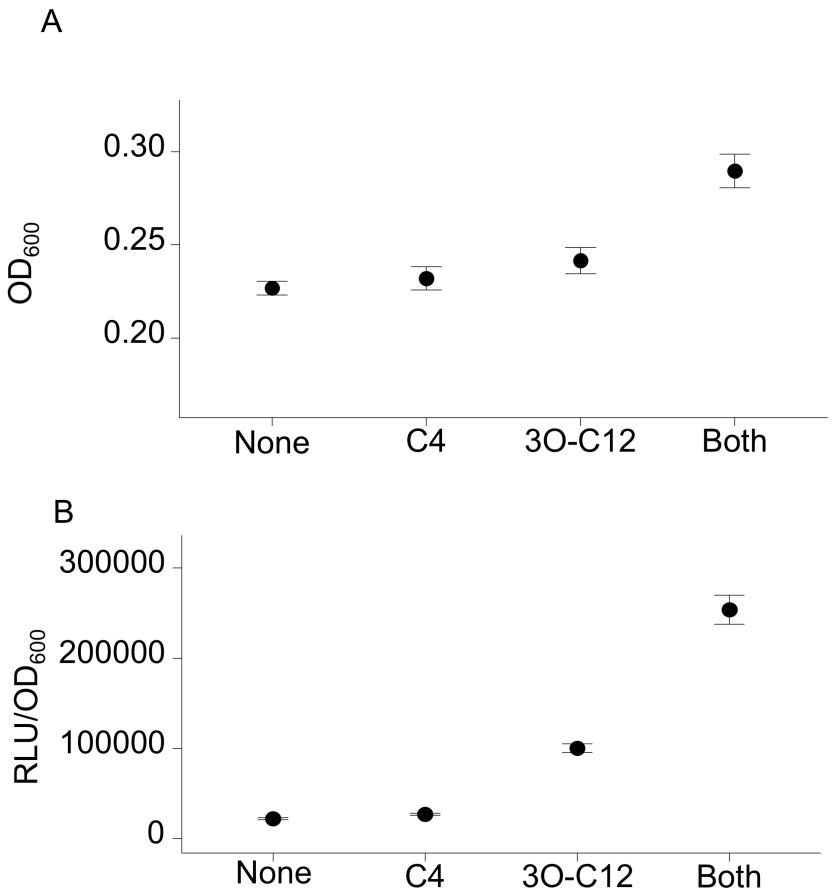

Figure 1. Combinatorial signaling (AND gate) determines *P. aeruginosa* growth and QS-dependent gene expression in QSM. A *P. aeruginosa* Δ*lasIrhlI* mutant (PAO-JG2) responds in a synergistic manner to both 3O-C12-HSL and C4-HSL signals with respect to (A) growth in QSM; (B) *lasB* expression in QSM. Each signal was added at a concentration of 0.5 μM. Error bars represent ± SEM of 5 replicate experiments.

Exploitation of combinatorial sensing allows for novel cheating strategies. Previous work has shown that *lasR* mutants act as social cheats in QSM by exploiting QS-cooperating cells that make public goods (proteases) cite1, 3, 13. Other work has demonstrated that the enrichment of *lasR* cheats can be controlled when QS links public goods production with private goods (adenosine) metabolism, by supplementing QSM with adenosine [9]. Combinatorial signaling theory predicts that secreted products (which can be socially exploited) are controlled synergistically by multiple signal molecules, and consistent with this we find that lasB expression is under AND-gate control (Figure 1A & B) [8]. In contrast, we found earlier that intracellular (private) adenosine metabolism by the purine nucleosidase Nuh (*nuh*) is under single signal control (a 3O-C12-HSL gate) [8]. Given these signal processing rules, a simple *lasR* cheat strategy (no response to 3O-C12-HSL) will profit when mixed with wildtype cells in QSM – but will pay a cost if growth is at all dependent on adenosine metabolism. We next asked whether bacteria can overcome this metabolic incentive to cooperate by evolving their signal processing rules to produce novel and more elaborate combinatorial cheat strategies.

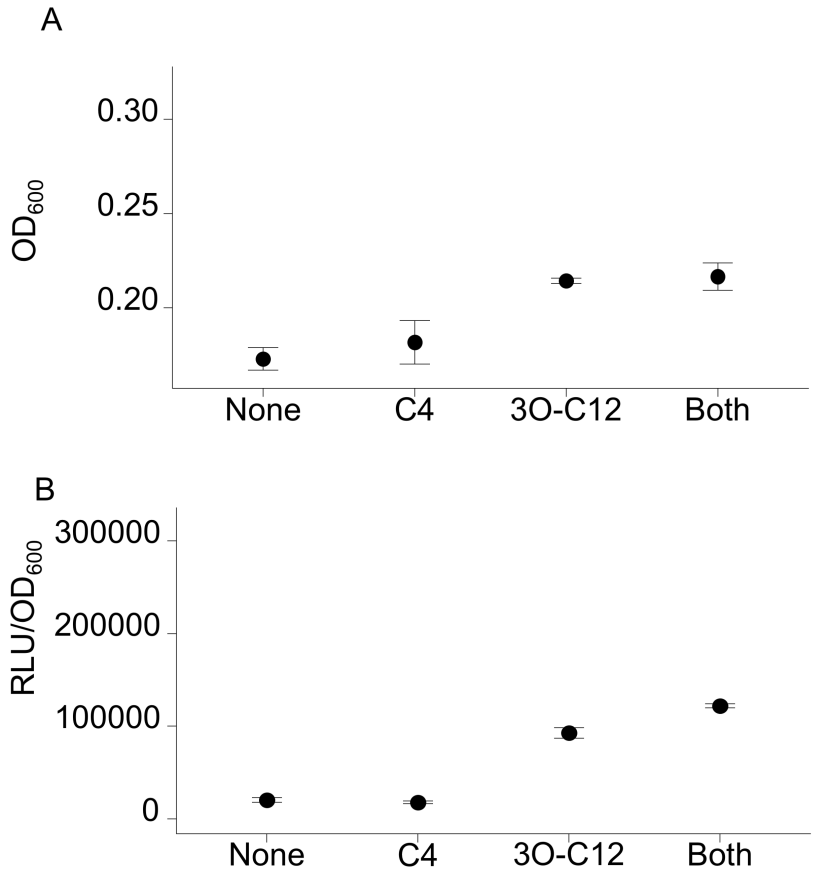

Figure 2. Combinatorial signaling is altered in an evolved isolate. The evolved mutant (NCRi) lost the ability to respond to both signals and neither growth or *lasB* expression was increased in QSM with the addition of both signals. Each signal was added at a concentration of 0.5 μM. Error bars represent $\pm$ SEM of 5 replicate experiments.

To test whether combinatorial cheats can evolve, we performed an evolution experiment (see Materials and Methods for details; Fig. S1). Briefly, our experiment involved cycling the bacteria between a private good-dependent growth media (adenosine) where activation via a 3O-C12-HSL gate is required for growth; and in a public good-dependent growth media (QSM) where activation of the AND-gate positively impacts growth. After 10 rounds of selection, we randomly selected a Non-Combinatorial Responding mutant (NCRi) that could respond to 3O-C12-HSL but had lost the ability to respond in a combinatorial manner to both 3O-C12-HSL and C4-HSL together. When grown in QSM as a monoculture, this mutant did not display any significant increases in growth (Figure 2 2A; $p = 0.\ 540$) or lasB expression (Fig. 2B; $p = 0.\ 631$) in QSM in the presence of both signals.

To test whether the evolved isolate could cheat on a strain that responds synergistically to two signals, we performed a competition experiment. We used an un-labeled PAO1Δ*lasI/rhlI* strain (PAO-JG1) [8] as the competing strain, to be able to distinguish between competing strains in co-culture. We also competed the PAO-JG2 ancestor against PAO-JG1. The only difference between PAO-JG1 and PAO-JG2 is that PAO-JG2 contains a *lasB::luxCDABE* fusion. We found that the fitness of PAO-JG2 compared to PAO-JG1 did not change when either 3O-C12-HSL alone or 3O-C12-HSL and C4-HSL together were added to competition experiments (Fig. 3). An expected result as these strains are essentially the same, with the only difference being the *lasB* reporter in PAO-JG2. We saw no difference in the relative fitness of NCRi compared to PAO-JG1 when 3O-C12-HSL was added in isolation (Fig. 3; $p = 0.702$). Under these conditions, both PAO-JG1 and NCRi grow to similar levels and express *lasB* to a similar extent (Figs. 1 and 2), and so it is likely that both are contributing similar QS responses and levels of public goods. In contrast, when both signals were added together, there was a significant increase in the relative fitness of NCRi (Figure 3; $p = 0.00015$). Under these conditions, the addition of both signals induces higher growth and *lasB* expression in the ancestor strain compared to NCRi due to a functional response to both signals. In this case, the mutant has reduced QS but still demonstrates increased fitness which is consistent with social cheating. This differs from previous cheating assays because the NCRi strain is exploiting the synergistic nature of public goods production.

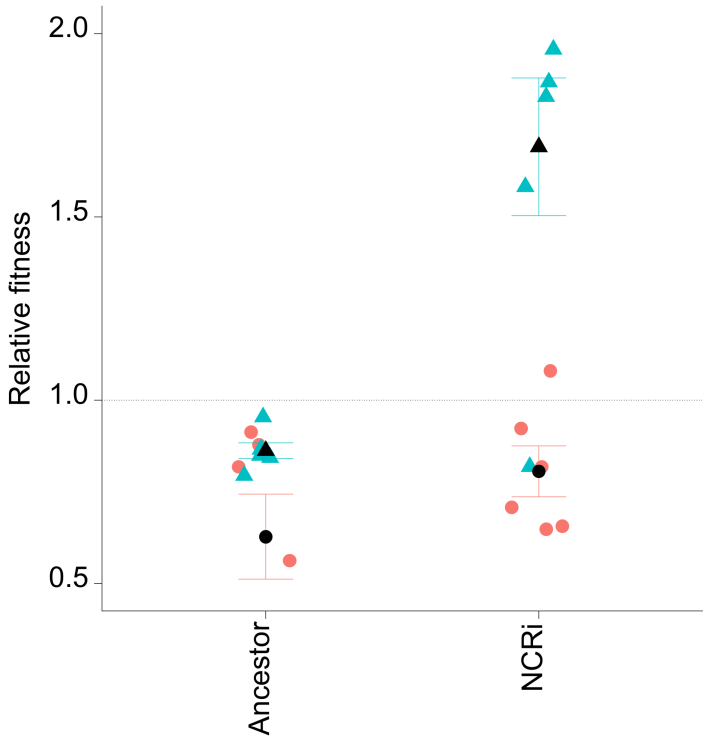

Figure 3. Cheating within combinatorial signaling. The evolved mutant (NCRi) relative fitness is increased when grown with a synergistically responding strain. Both PAO-JG2 (ancestor) and NCRi were competed against a non-*lux* labelled PAO1Δ*lasI/rhlI* strain (PAO-JG1). PAO-JG2 and NCRi each began at a 1:10 frequency with PAO-JG1, with either the addition of 0.5 μM 3O-C12-HSL alone (Red circles) or 0.5 μM 3O-C12-HSL & C4-HSL in combination (Blue triangles). The labelled ancestor strain (PAO-JG1) showed no significant increases in fitness compared to the unlabeled PAO-JG1 in all treatments. The evolved strain NCRi demonstrated a significant increase in relative fitness when both signals were added in combination. Error bars are ± SEM with 6 replicates The black spot is the medium value.

The loss of combinatorial response in NCRi is not due to mutations in known QS genes. Whole genome sequencing of NCRi detected no mutations in genes previously shown to be involved in the QS-regulatory cascade in *P. aeruginosa*. Importantly, the regulators *lasR, rhlR, and pqsR* were all-intact and identical when compared to the ancestor strain and the PAO1 reference library. Sequencing showed a single polymorphism in the gene *mexF* and probable deletions of 4 genes; *PA0713* a hypothetical protein; *ndvB* which regulates the production of glucans that sequester aminoglycosides and is linked to membrane permeability [28]; and *PA1193* a predicted DAM glycosylase. How these mutations impact on combinatorial signaling remains unknown, however, the lack of mutation in known QS-regulated genes, suggests novel ways to circumvent the known QS system in *P. aeruginosa*, which allows strains to evolve to bypass the restrictions levied by QS-private goods metabolism.

Complementation of *rhlR* did not restore the combinatorial response. Our sequencing of NCRi showed that the major regulators of QS were intact, suggesting possible indirect effects on the QS system. As we found that NCRi lost the ability to

respond to C4-HSL but not 3O-C12-HSL, we complemented the strain with a constitutively expressed *rhlR* gene. This had a minimal effect on *lasB::lux* expression and did not enable NCRi to respond synergistically (Figure 4; p ¡ 0.0000001).

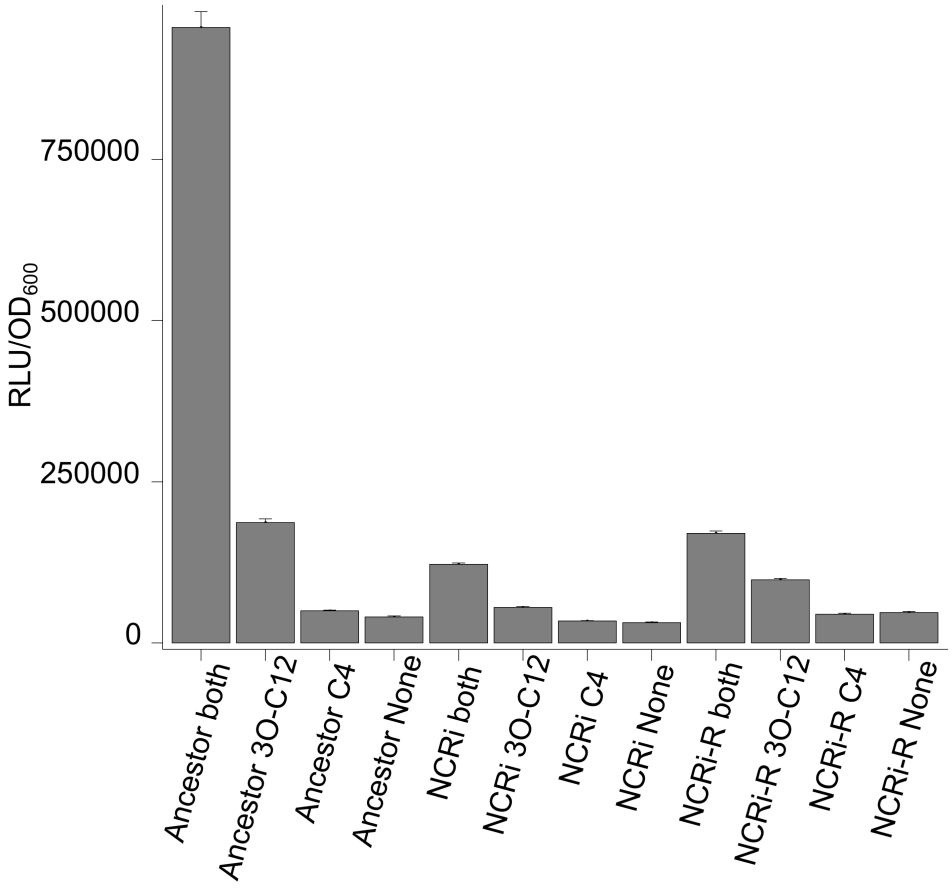

Figure 4. Complementation with RhlR did not restore synergistic response. NCRi was complemented with *rhlR* on a constitutive promoter (NCRi-R) to determine whether indirect effects on *rhlR* were due to lack of synergistic response. Complementation increased the response to all treatments including the control but a synergistic response was not seen. Signals added at 5 μM each, error bars are ± SEM with 5 replicates.

Selection for reduced cooperation increased resistance to chloramphenicol. The *mexF* gene is partly responsible for the efflux of antibiotics such as chloramphenicol [19]. Recent work has also shown that the MexCD [2] and MexEF (Ron D. Oshri, Keren S. Zrihen, Itzhak Shner, Shira Omer Bendori and Avigdor Eldar submitted for publication) efflux systems are involved in QS signal levels and response to signals. We found that the MIC to chloramphenicol was significantly increased in NCRi compared to the ancestor strain. PAO-JG2 had an MIC of 85 $\mu g^{\text{-}\mu l}$ while NCRi had an MIC of 480 $\mu g^{\text{-}\mu l}$ (table 1; $p = 0.019$).

Table 1. NCRi-R displays increased resistance to chloramphenicol. NCRi has a SNP in *mexF*, an efflux pump partly responsible for the efflux of the antibiotic chloramphenicol and recently implicated in regulating the QS response. The MIC of the ancestor and evolved isolate NCRi to chloramphenicol were determined by serial dilution in a range of concentrations from 0-1600 $\mu g^{\text{-}\mu l}$. Bacteria were initially cultured at an OD_{600} of 0.05 and the MIC was determined to be an concentration in which growth did not exceed an OD_{600} of 0.1 after 18 hours. Error bars are ± SEM of 5 replicates.

Strain	MIC $\mu g^{\text{-}\mu l}$	± SEM
Ancestor	85	10
NCRi-R	480	80

Discussion

A rapidly growing body of research has demonstrated that bacterial QS is a social behavior that is exploitable by cheats [8, 12, 13, 21, 22, 26, 33, 35–37], and a number of explanations for maintaining QS in natural populations have been provided. Kin selection has previously been shown to maintain QS in populations because it increases the reproductive success of producer cells (direct fitness) and other individuals that carry the same QS genes (indirect fitness) [12, 13, 16, 17, 20, 39]. The effect of kin selection on QS relies on the fact that QS controls public goods production (such as exoproteases) that can be shared locally between cells [10, 12, 29]. We are now becoming increasingly aware that the QS system does not just regulate the production of public goods, but it also controls private goods metabolism within cells. By linking QS to both public and private goods, it has been shown that this can prevent the invasion of public goods cheats [8–10, 24]. This has been termed a 'metabolic incentive to cooperate' [9].

Here we describe a selection experiment (Fig. S1) specifically designed to test whether metabolic incentives for cooperation can be circumvented by cheats. We based our experimental approach on a number of previous findings. Firstly, that QS-dependent exoprotease production is required for maximal growth in a medium containing a carbon source that is degraded by proteases which act as public goods [10, 12, 29]. Secondly, that expression of the *lasB* gene required for protease (elastase) production is synergistically increased by the addition of C4-HSL and 3O-C12-HSL in combination [8, 34, 38]. Thirdly, that the enrichment of *lasR* cheats can be controlled by private goods metabolism such as growth in adenosine [9, 30].

Our findings show that there are fitness benefits in using a combination of C4-HSL and 3O-C12-HSL to regulate public goods that break down a protein carbon source in QSM (Fig 1A). Many factors could impact on the combinatorial response. Shifts in mass transfer, the total level of rich carbon source, the spatial structure of the population, and levels of potential microbial crosstalk, will almost certainly change the benefits of QS fitness of cells responding in a combinatorial manner [8, 10]. Fitness benefits provided by combinatorial signaling, therefore, opens the door for conditional cheats that can access

QS-dependent public goods with lower production of metabolically costly proteases.

We identified an evolved isolate (NCRi) that circumvents the QS-dependent metabolic incentives to cooperate. This isolate acts as a social cheat and has a higher fitness in competition with its ancestor, and disruption of combinatorial signaling was not directly linked to mutations in the known QS cascade of *P. aeruginosa*. The response of NCRi to signal treatments showed that it is capable of using 3O-C12-HSL in isolation to make public goods to sequester carbon, but it lost the synergistic (AND-gate) response when exposed to both 3O-C12-HSL and C4-HSL.

We found that this loss of the ancestral synergistic response resulted in the mutant having a fitness advantage in the presence of both signals (Fig. 3). Although both strains are capable of producing public goods, indicated by the expression of the *lasB::lux* reporter in the presence of 3O-C12-HSL; the mutant lacks the synergistic response; and will therefore produce less public goods. This suggests that in an environment in which QS cooperation is required for maximal fitness, NCRi receives higher fitness benefits when in competition with the ancestor, because individuals that have maintained the synergistic response to the signals will be producing a greater amount of public goods than strains that have rewired their combinatorial responses to respond only to 3O-C12-HSL [10, 12, 29].

A simple mechanistic explanation for the lack of response to C4-HSL would be a defect in the *rhl* QS system; however, when we whole genome sequenced NCRi, we found no changes in the *rhl* genes or a range of known QS genes (including *pqs* genes) that could easily explain our observed phenotype. To determine whether indirect effects on the *rhl* system were responsible for the NCRi phenotype, we added the *rhlR* gene on a constitutive promoter into NCRi. We found that constitutive *rhlR* expression did not restore the combinatorial response in NCRi, suggesting that the response to both signals may be regulated through non-canonical QS pathways.

The sequencing results flagged mutations in *mexF* and *ndvB*. Mex pumps have been linked to QS regulation [1] and the *ndvB* gene has been shown to control the production of glucans associated with the biofilm matrix of *P. aeruginosa* and to provide a structural correlation between biofilm production and antibiotic resistance (23, 34) . NdvB is reportedly linked to membrane permeability which could affect QS signal concentrations within cells [14, 28]. Recent work (Ron D. Oshri, *et al*, submitted for publication) has shown that a *mexT* mutation leads to repression of the MexEF-OprN system increasing QS cooperation. This is because repression of this efflux system leads to greater activation of the *Rhl* QS system, presumably by reducing efflux of C4-HSL across the membrane and increasing the effective internal concentration. This repression also reduced resistance to chloramphenicol. Our results are coherent with this finding as NCRi has a reduced response to QS molecules and an increased resistance to chloramphenicol, suggesting the SNP in *mexF* may increase efflux and reduce internal signal concentration. Such an effect would account for the reduced QS response and increased chloramphenicol resistance.

The findings presented here and work by others suggests that many selective pressures work together to control cheats in natural environments. Factors such as migration, spatial structure, regulatory responses and private goods metabolism could all function together to reduce the relative fitness of cheats [8]. Our work shows that the incentives to cooperate placed by metabolic sources are not adequate to explain the persistence of cooperation in *P. aeruginosa*. Combinatorial responses allow for a greater range of social strategies to emerge than previously considered. Further our results should be taken as preliminary evidence that there remain novel regulators of QS in *P. aeruginosa*, that are capable of regulating protease production and presumably other QS-controlled genes.

Supporting Information

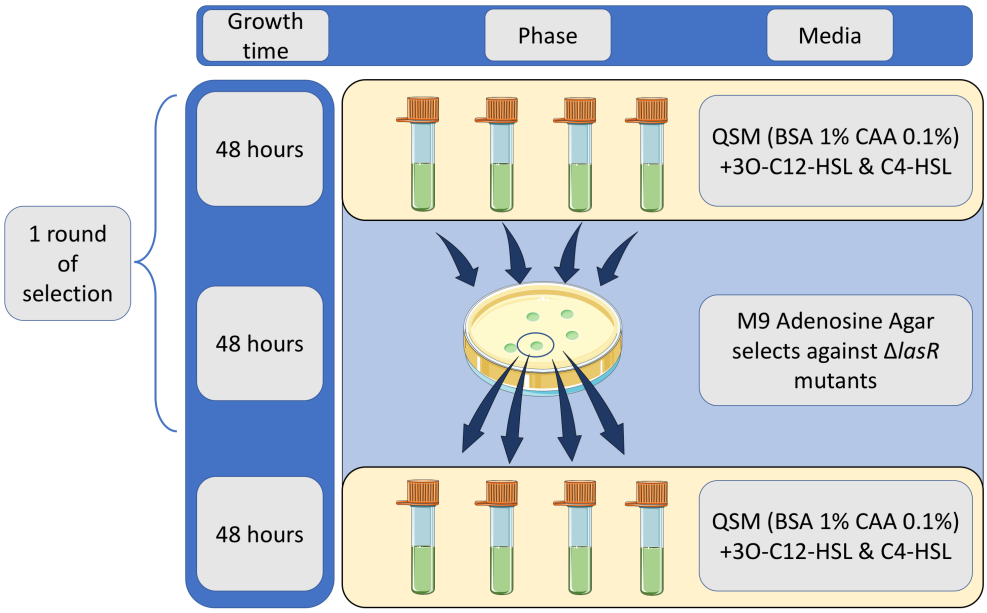

Figure S1. Schematic of selection experiment. Our selection experiment was performed using two separate phases of growth. (1) A liquid phase in which BSA would be broken down by the production of public good enzyme, extracellularly, and thus open to exploitation of individuals producing lower levels of protease. (2) A phase on agar plates where acquisition of carbon was achieved by the intracellular process of adenosine utilization, which requires a functional las QS via 3O-C12-HSL. The selection experiment also used a global selection approach to maintain cooperation. In each replicate line, 4 growth microcosms were pooled at the end of the liquid phase and streaked onto a single adenosine plate, thus promoting the selection of the overall best growers.

Acknowledgments

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