ORIGINAL ARTICLE

Non-social adaptation defers a tragedy of the commons in *Pseudomonas aeruginosa* quorum sensing

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In a process termed quorum sensing (QS), the opportunistic bacterial pathogen *Pseudomonas aeruginosa* uses diffusible signaling molecules to regulate the expression of numerous secreted factors or public goods that are shared within the population. But not all cells respond to QS signals. These social cheaters typically harbor a mutation in the QS receptor gene *lasR* and exploit the public goods produced by cooperators. Here we show that non-social adaptation under growth conditions that require QS-dependent public goods increases tolerance to cheating and defers a tragedy of the commons. The underlying mutation is in the transcriptional repressor gene *psdR*. This mutation has no effect on public goods expression but instead increases individual fitness by derepressing growth-limiting intracellular metabolism. Even though *psdR* mutant populations remain susceptible to invasion by isogenic *psdR lasR* cheaters, they bear a lower cheater load than do wild-type populations, and they are completely resistant to invasion by *lasR* cheaters with functional *psdR*. Mutations in *psdR* also sustain growth near wild-type levels when paired with certain partial loss-of-function *lasR* mutations. Targeted sequencing of multiple evolved isolates revealed that mutations in *psdR* arise before mutations in *lasR*, and rapidly sweep through the population. Our results indicate that a QS-favoring environment can lead to adaptations in non-social, intracellular traits that increase the fitness of cooperating individuals and thereby contribute to population-wide maintenance of QS and associated cooperative behaviors.

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Introduction

Bacterial cell–cell signaling, termed quorum sensing (QS), often coordinates other cooperative behaviors such as nutrient acquisition, biofilm formation or virulence in a cell density-dependent manner (Waters and Bassler, 2005; Williams *et al.*, 2007). In Gram-negative proteobacteria, QS generally comprises a LuxI-type signal synthase that produces a diffusible acyl-homoserine lactone (acyl-HSL) signal, and a cognate LuxR-type receptor that binds the signal and regulates transcription of target genes (Waters and Bassler, 2005; Williams *et al.*, 2007; Schuster *et al.*, 2013). The opportunistic pathogen *Pseudomonas aeruginosa*, a particularly well-understood example, employs two acyl-HSL signaling systems, LasI/R and RhII/R, arranged in a hierarchical manner with LasR sitting atop the hierarchy (Schuster and Greenberg, 2006; Williams and Camara, 2009; Jimenez *et al.*, 2012). Together, both

systems regulate over 300 genes, many of which encode secreted public goods such as extracellular enzymes or secondary metabolites that have a role in virulence (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

How social behaviors such as QS evolve and are maintained is of intense research and debate, as exploitation of common resources by selfish individuals should be favored and lead to a so-called ‘tragedy of the commons’ (Keller and Surette, 2006; West *et al.*, 2006). A tragedy of the commons results when the magnitude of selfish exploitation by cheaters exceeds the capacity of a cooperative system, resulting in the collapse of the entire population. Indeed, several studies have demonstrated the emergence of QS cheaters that reap the benefits of cooperative secretions without metabolic investment both *in vitro* (Diggle *et al.*, 2007; Sandoz *et al.*, 2007; Wilder *et al.*, 2011; Dandekar *et al.*, 2012) and *in vivo* (Kohler *et al.*, 2009; Rumbaugh *et al.*, 2009). These QS cheaters are defined by a loss-of-function mutation in the gene coding for the primary QS receptor LasR. We previously showed that *P. aeruginosa lasR* mutant cheaters consistently evolve in a minimal growth medium with casein as the sole carbon source that requires QS-dependent extracellular proteolysis (Sandoz *et al.*, 2007).

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Using defined wild-type and *lasR* mutant co-cultures, we further showed that these cheaters do better when they are rare (that is, display negative frequency-dependent fitness), and that they impose a burden on population growth (Sandoz *et al.*, 2007; Wilder *et al.*, 2011). Intriguingly, however, this negative effect on group fitness was generally not observed during *in vitro* evolution experiments initiated solely with the wild-type strain, suggesting evolution of a mechanism that stabilized QS (Sandoz *et al.*, 2007; Wilder *et al.*, 2011; Dandekar *et al.*, 2012).

To identify and characterize the underlying mechanism, we used a combination of whole-genome sequencing, genetic analysis and growth experiments. We found a single mutation in a transcriptional repressor, *PsdR*, that rapidly dominates the population, enhances intracellular dipeptide metabolism, increases both individual and group fitness, provides immunity against cheaters that do not themselves carry a *psdR* mutation and lessens the detrimental effect of certain *lasR* mutations on group fitness. Our results show that QS-favoring conditions can select for non-social adaptations that improve group fitness and defer a tragedy of the commons.

Materials and methods

Strains and culture conditions

P. aeruginosa PAO1 was used as the wild-type isogenic parent at the start of all original *in vitro* evolution experiments (Sandoz *et al.*, 2007; Wilder *et al.*, 2011). All mutants were created via allelic exchange using a suicide vector containing either evolved alleles or in-frame deletions constructed by splicing-overlap-extension PCR (Horton, 1995; Hoang *et al.*, 1998) (see Table 1 for a comprehensive list of strains). For routine culturing, we grew strains at 37 °C on Lennox lysogeny broth (LB) agar or with shaking in Lennox LB broth buffered with 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.0. Plates were supplemented with 100 µg ml⁻¹ tetracycline when necessary for the selection of marked strains. For fitness, competition, substrate utilization and expression assays, M9 minimal medium supplemented with either 1% caseinate, 0.5% casamino acids (CAA) or 10 mM GlyGlu dipeptide was used (Sandoz *et al.*, 2007; Kiely *et al.*, 2008). In the case of caseinate fitness experiments with supplemented exoprotease, porcine elastase (Sigma, St. Louis, MO, USA) was added at the beginning of growth, in principle as described previously (Diggle *et al.*, 2007). As determined with a fluorescein isothiocyanate (FITC)-casein assay (see below), the caseinolytic activity of the elastase concentration used (0.21 U ml⁻¹) was 20% of that found in the supernatant of wild-type cultures grown in M9-caseinate medium for 24 h. All experiments were performed using a minimum of three biological replicates with independent inocula.

Whole genome sequencing and targeted DNA sequencing

For genome sequencing, we selected an evolved 'hybrid cooperator' (HC) isolate from our previous long-term growth experiment (Sandoz *et al.*, 2007) and its wild-type PAO1 parent strain. The evolved isolate was dubbed HC because of its partially positive QS phenotypes (see the Results section for details). Both strains were grown individually overnight (18 h) in MOPS-buffered LB medium as described above. Genomic DNA was isolated using the Qiagen Puregene Yeast/Bacteria Kit B (Qiagen Sciences, Germantown, MD, USA) and assessed for quality on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The 454 pyrosequencing was carried out using unpaired reads on a Genome Sequencer FLX instrument with GS FLX Titanium series reagents (454 Life Sciences, Branford, CT, USA) by the Dhingra Genomics Lab at Washington State University in Pullman, Washington, USA. Sequencing of the HC isolate produced 507 094 reads covering ~187 Mb, whereas the ancestral PAO1 produced 501 270 reads covering ~200 Mb. Raw 454 reads were assembled using the Roche 454 Newbler assembler (Roche Diagnostics, Basel, Switzerland) with the PAO1 genome as a reference (Stover *et al.*, 2000; Margulies *et al.*, 2005; Winsor *et al.*, 2011). The HC assembly utilized an average map length of 370 bp and average sequence depth of 29.5, whereas the ancestral PAO1 assembly utilized an average map length of 401 bp and average sequence depth of 31.7. Differences between the HC and ancestral PAO1 assemblies were discovered using SNP/INDEL calling in SAMtools (Li *et al.*, 2009). To confirm the identified mutations, and to sequence specific loci of interest, standard dideoxy sequencing of PCR-amplified and purified chromosomal DNA was employed at the Center for Genome Research and Biocomputing at Oregon State University in Corvallis, OR, USA. Primers are listed in Supplementary Table S1.

Fitness assays and cheater load

Wild-type, evolved HC and defined mutants (tagged with an antibiotic-resistance marker where applicable) were grown in caseinate minimal medium. Overnight cultures of individual strains in MOPS-buffered LB were used as inocula for experiments and diluted to starting OD₆₀₀ values of 0.02 (1 cm path length, ~2 × 10⁷ colony-forming units (CFUs) per ml). In the case of co-culture experiments, the combined total starting OD₆₀₀ was 0.02. All fitness experiments were allowed to proceed for 24 h with shaking at 37 °C. For rich media (LB + MOPS, M9-CAA) and dipeptide media co-cultures, conditions were kept identical to caseinate experiments with the exception that co-cultures in dipeptide media were grown for 7 days to allow the cultures to reach saturation. CFUs per ml were determined by dilution plating at *t* = 0 and 24 h, with an additional plating

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or origin
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild-type (obtained from M Vasil and U Ochsner)	Holloway (1955)
PAO-HC	PAO1 derivative; evolved hybrid cooperator containing <i>lasR5</i> , <i>psdR1</i> and <i>abcB1</i> mutations	Sandoz <i>et al.</i> (2007)
PAO1 <i>lasR5</i>	PAO1 derivative; <i>lasR5</i> , unmarked mutant in which wild-type <i>lasR</i> was replaced with <i>lasR5</i>	This study
PAO1 <i>psdR1</i>	PAO1 derivative; <i>psdR1</i> , unmarked mutant in which wild-type <i>psdR</i> was replaced with <i>psdR1</i>	This study
PAO1 Δ <i>lasR</i>	PAO1 derivative; Δ <i>lasR</i> , unmarked in-frame deletion from amino acids 102–216	Wilder <i>et al.</i> (2011)
PAO1 Δ <i>psdR</i>	PAO1 derivative; Δ <i>psdR</i> , unmarked in-frame deletion from amino acids 11–124	This study
PAO1 <i>psdR1 lasR5</i>	PAO1 <i>psdR1</i> derivative; <i>psdR1 lasR5</i> , unmarked mutant in which wild-type <i>psdR</i> and <i>lasR</i> were replaced with <i>psdR1</i> and <i>lasR5</i> , respectively	This study
PAO1 <i>psdR1</i> Δ <i>lasR</i>	PAO1 Δ <i>lasR</i> derivative; <i>psdR1</i> Δ <i>lasR</i> , unmarked mutant in which wild-type <i>psdR</i> and <i>lasR</i> were replaced with <i>psdR1</i> and Δ <i>lasR</i> , respectively	This study
<i>Escherichia coli</i>		
DH5 α	F- Φ 80lacZYA-argF U169 recA1 hsdR17 (rk [−] , mk ⁺) phoA supE44 λ -thi-1 gyrA96 relA1	Invitrogen
SM10	thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λ pir	Simon <i>et al.</i> (1983)
<i>Plasmids</i>		
pEX18Gm	Conjugative suicide plasmid; Gm ^R	Hoang <i>et al.</i> (1998)
pEX18Gm. <i>psdR1</i>	pEX18Gm with the evolved <i>psdR1</i> allele	This study
pEX18Gm. Δ <i>psdR</i>	pEX18Gm with Δ <i>psdR</i> containing an in-frame deletion from amino acids 11–124	This study
pEX18Gm. <i>lasR5</i>	pEX18Gm with the evolved <i>lasR5</i> allele	This study
pUC18R6KT-mini-Tn7T-Tet	Broad host range mini-Tn7 vector with Tc resistance gene cassette	Courtesy of Herbert P Schweizer

at $t = 12$ h during absolute fitness experiments. For enumeration in co-culture experiments, differential plating on tetracycline-supplemented LB agar was used. Fitness was calculated according to the Malthusian growth model (Lenski *et al.*, 1991; Wilder *et al.*, 2011). Absolute fitness is expressed as the average rate of increase or Malthusian parameter (m), with $m = \ln(N_1/N_0)/t$, where N_1 and N_0 are the final and initial strain densities, respectively, and t is the culturing time in days. Relative fitness is expressed as the ratio of the Malthusian parameters (w) of two competing strains. Cheater-load experiments were performed as previously described (Sandoz *et al.*, 2007), with the exception that for this set of experiments total starting OD₆₀₀ values of 0.02 were identical for all treatments.

Extracellular proteolysis

Extracellular caseinolytic activity was determined using an established FITC-casein assay (Twining, 1984; Wilder *et al.*, 2011). Briefly, starter cultures of each strain were grown overnight in MOPS-buffered LB at 37 °C and diluted to an OD₆₀₀ of 0.02 in fresh CAA medium. Supernatants were harvested after 12 h of growth, sterile filtered and incubated with the FITC-conjugated casein substrate (Sigma). Digestion was allowed to proceed for 3 h at 37 °C. Fluorescence was measured at $\lambda_{\text{ex}} = 490$ nm and

$\lambda_{\text{em}} = 525$ nm in a 96-well format on a Tecan Infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland).

To predict the cleavage pattern of our caseinate substrate (a mixture of α -s1-casein, α -s2-casein, β -casein and κ -casein) by LasB elastase, we employed ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter/) for *in silico* digestion with thermolysin, the closest LasB elastase family member available in the database (Gasteiger *et al.*, 2005).

Expression analysis

Strains were initially grown overnight in MOPS-buffered LB liquid culture, and then diluted to an OD₆₀₀ of 0.02 in fresh CAA medium. Expression cultures were harvested at OD₆₀₀ values of 0.5 and 1.5, corresponding to exponential and early stationary phases in this medium, respectively. Total RNA was isolated and complementary DNA synthesized as previously described (Schuster and Greenberg, 2007; Schuster, 2011). Quantitative reverse-transcriptase PCR was carried out according to established protocols (Schuster and Greenberg, 2007; Schuster, 2011) using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Identical amounts of complementary DNA were used as template. Transcript levels were quantified using the relative standard curve method.

Results

Genome sequencing of *in vitro*-evolved *P. aeruginosa*

In our previous *in vitro* evolution studies, we cultured the PAO1 wild-type strain in caseinate medium for 20 days, subculturing into fresh medium each day (Sandoz *et al.*, 2007; Wilder *et al.*, 2011). We used two phenotypic screens as a proxy for QS proficiency, namely (1) protease production on skim-milk agar plates and (2) growth on minimal agar plates with adenosine as the sole carbon source. Negative results in both phenotypic screens correspond to mutations in the gene coding for the primary QS regulator, *lasR*, thereby conferring a cheater phenotype.

To confirm that QS-controlled extracellular proteolysis is solely responsible for the growth deficiency of the pleiotropic *lasR* mutant in caseinate medium, we cultured the $\Delta lasR$ single mutant in the presence of purified elastase. Addition of elastase restored $\Delta lasR$ mutant fitness, expressed as Malthusian growth parameter, m (Lenski *et al.*, 1991), to a level indistinguishable from wild-type and significantly above that of the $\Delta lasR$ mutant without elastase (Figure 1).

Although cheaters can exploit the public goods produced by the cooperating population in a way that eventually leads to a population crash, we only observed this outcome in one of our five replicate evolution experiments (Figure 2a–f). Instead, we found that *lasR* cheater frequencies of 25% on average are tolerated and do not significantly affect the growth yield of the population (Figure 2a). This was surprising because our previous co-culture experiments with specific initial frequencies of defined *lasR*-mutant cheaters and wild-type cooperators showed that comparable levels of

cheaters impose a substantial burden on the productivity of a population (Sandoz *et al.*, 2007).

We also observed a subpopulation of isolates deficient in growth on adenosine, but not in skim-milk proteolysis. Based on their phenotypes, we dubbed these isolates HCs. The HC subpopulation rose in frequency similar to the cheaters, representing up to 20% of the total population (Figure 2a), and also harbored mutations in *lasR* (Sandoz *et al.*, 2007). We reasoned that this HC phenotype had an important role in the maintenance of cooperative population growth. We hypothesized that the HC phenotype was caused by an independent second-site mutation that occurred either before or after mutation of *lasR*, and that this mutation partially restored QS proficiency.

To address our hypothesis, we sequenced the genome of a representative HC isolate from day 12 of one replicate experiment. We also sequenced the genome of the ancestral PAO1 wild-type strain for comparison. Genomes were assembled using the published PAO1 genome sequence as a reference (Stover *et al.*, 2000; Winsor *et al.*, 2011). In all, our analysis showed the HC harbored only three mutations when compared with the wild-type ancestor, including *lasR*. The mutations were single-nucleotide polymorphisms in *lasR* (PA1430) and in PA2408, as well as an 18 base-pair truncation in *psdR* (PA4499). PA2408 encodes a probable adenosine triphosphate (ATP)-binding component of an ABC (ATP-binding cassette) transporter, and *psdR* encodes a transcriptional regulator (Kiely *et al.*, 2008; Winsor *et al.*, 2011) (Table 2). Targeted Sanger sequencing of all three loci in two additional HC and cheater isolates from day 12 of two independent *in vitro* evolution experiments revealed that mutations in *lasR* and *psdR* are ubiquitous in isolates displaying both phenotypes, but mutations in PA2408 are not (Supplementary Table S2). We therefore concluded that the PA2408 mutation is not likely to be relevant to the HC phenotype. Additional evidence for this conclusion is presented below.

The presence of *psdR* mutations in cheater isolates in addition to the HC isolates indicated that a mutation in *psdR* is not a distinguishing feature of the HC phenotype, and that mutation of *psdR* may have arisen before mutation of *lasR*. To elucidate the evolutionary trajectories of the *psdR* mutation, and to assess whether *psdR* mutations are also present in cooperator phenotypes, we sequenced the *psdR* locus of evolved isolates positive for both skim-milk proteolysis and adenosine utilization from the day 4, 8 and 12 archives of two replicates (5 isolates per day, 30 total). Surprisingly, we found 100% of sequenced isolates harbored a nonsynonymous mutation in *psdR* (Table 3). Even as early as the first phenotypic screen at day 4, the entire sampled population that originally appeared to be ‘wild-type’ with respect to QS actually had acquired point mutations, insertions or deletions in *psdR*. Isolates

Figure 1 Effect of elastase addition on the absolute fitness of a *lasR* mutant. Absolute fitness of the *P. aeruginosa* $\Delta lasR$ mutant and its wild-type parent was calculated as Malthusian growth parameter (m) after 24 h of growth in caseinate medium. In the case of $\Delta lasR$ + elastase, 0.21 U ml^{-1} porcine elastase was added. Bars represent means ($n=3$). *Significant difference as determined by unpaired *t*-test ($P=0.00010$). NS, difference not significant (unpaired *t*-test, $P=0.096$).

Figure 2 *In vitro* evolution of *P. aeruginosa* populations under conditions that require QS. (a–f) Population growth yield and phenotypic frequencies. OD₆₀₀ values measured daily before subculture are plotted on the left vertical axis (blue line). Frequencies of cooperator (black triangles), cheater (red circles) and hybrid cooperator (green squares) phenotypes are plotted on the right vertical axis. (a) Means and s.e.m. of all replicate experiments (*n* = 5). In some cases, error bars are too small to be seen. (b–f) Individual, independent biological replicates. Based on raw data from Wilder *et al.* (2011; b, c) and Sandoz *et al.* (2007; d–f). (g) Schematic of evolutionary trajectories of individual mutations.

from the same replicate culture often harbored different *psdR* mutations, and some *psdR* mutations in early cooperator isolates were identical to those in

later HC and cheater isolates. These results therefore suggest that the HC phenotype is primarily defined by the nature of the *lasR* mutation itself. In general, our

Table 2 Mutations in a sequenced *Pseudomonas aeruginosa* hybrid cooperator (HC) genome

Gene (name) ^a	Function ^a	Mutation ^b	Allele ID
PA1430 (<i>lasR</i>)	LuxR-type transcriptional regulator	C→T (683)	<i>lasR5</i>
PA4499 (<i>psdR</i>)	Putative transcriptional regulator	Δ18 bp (514)	<i>psdR1</i>
PA2408	Probable ATP-binding component of ABC transporter	T→C (337)	<i>abcB1</i>

^aGene names and functions as annotated in the *Pseudomonas* Genome Database.^bNumbers in parentheses indicate position or beginning of a given mutation relative to the translational start site.**Table 3** The *psdR* mutations in evolved *Pseudomonas aeruginosa* isolates

Mutations ^a	Number of mutations (replicate) ^b			Change ^c
	Day 4	Day 8	Day 12	
<i>Cheater (4 sequences total)</i>				
Δ505-end			2 (2)	Deletion
T166C			2 (1)	S56P
<i>HC (4 sequences total)</i>				
Δ145–148			2 (2)	Deletion
Δ261–422			1 (1)	Deletion
Δ261-end			1 (1)	Deletion
<i>Cooperator (30 sequences total)</i>				
Δ261–422	1 (1)	1 (2)		Deletion
Δ505-end		1 (2)		Deletion
Δ147–159		1 (1)	1 (1)	Deletion
C74T	1 (2)	1 (2)		A25V
T100C	1 (2)			F34L
C109A	1 (1)		1 (1)	Q37K
T166C	1 (1)			S56P
G397A	2 (1)	2 (1)		G133R
C411A			3 (1)	STOP at 137
A431C	1 (2)	1 (1)	2 (2)	Y144S
Insert A at 3		1 (1)		Frameshift
Insert A at 378	1 (2)	1 (2)	1 (2)	Frameshift
No amplicon	1 (2)	1 (2)	2 (2)	Unknown

^aMutations are sorted by cheater, hybrid cooperator (HC) and cooperator phenotypes. Numbers indicate nucleotide position or beginning of a given mutation relative to the translational start site.^bThe individual replicate of the *in vitro* evolution experiment is indicated in parentheses. Same-day isolates with identical mutations were always from the same replicate. Replicates 1 and 2 correspond to Figures 2b and c, respectively.^cNumbers indicate amino-acid position relative to the translational start site.

sequencing data indicate a strong selection against a functional *psdR* during cooperative growth in caseinate medium, and show that the evolutionary trajectories of cheater, HC and cooperator phenotypes all start with a mutation in *psdR* (Figure 2g). This result is consistent with the presumed function of PsdR. PsdR has been characterized as a transcriptional repressor of genes involved in the uptake and intracellular degradation of dipeptides in *P. aeruginosa* (Kiely et al., 2008). Thus, derepression of dipeptide metabolism through mutation and inactivation of PsdR could potentially increase the fitness of *P. aeruginosa* during proteolytic growth in caseinate medium. Such a mutant would take up and process the

dipeptides generated by the cocktail of secreted proteases (including LasB elastase, alkaline protease and protease IV) more rapidly. Consistent with this idea, *in silico* digestion of bovine casein by thermolysin, a homolog of *P. aeruginosa* LasB elastase with similar cleavage properties (Moriwaka and Tsuzuki, 1971; Jiang and Bond, 1992), indeed produces up to 6 dipeptides per casein molecule.

Absolute fitness of evolved and defined strains

Next, we investigated the fitness contributions of each mutant allele to the cooperator, cheater and HC phenotypes. We constructed defined single and double mutants by transferring the evolved *lasR5* and *psdR1* alleles into the parental PAO1 strain background, and compared their phenotypes with those of in-frame deletion mutants. This approach also allowed us to assess whether the nature of the mutation in *lasR* distinguishes a HC from a cheater. As characterized in our previous study, all evolved cheaters deficient in skim-milk proteolysis and adenosine utilization were also fully deficient in other QS-dependent phenotypes, identical to a *lasR* in-frame deletion mutant (Sandoz et al., 2007).

We first assessed growth of individual strains by measuring their population densities during clonal growth in caseinate medium that requires QS-dependent proteolysis (Figure 3a). The corresponding absolute fitness values and statistical data are shown in Supplementary Figure S1. The wild-type is capable of two logs of growth within 24 h, from $\sim 10^7$ to 10^9 CFUs per ml, whereas a *lasR* deletion mutant shows little growth. Interestingly, the defined *lasR5* mutant displayed an intermediate level of growth and fitness significantly above that of the $\Delta lasR$ mutant at 24 h, indicating that *lasR5* retains partial function. The defined *psdR1* mutant and the $\Delta psdR$ mutant displayed similar growth and fitness levels significantly above that of the wild-type at 12 h, indicating that the *psdR1* mutation completely inactivates gene function. The defined *psdR1 lasR5* double mutant and the evolved HC showed identical growth characteristics, similar to that of the wild-type at 24 h, supporting our sequencing data suggesting that the PA2408 mutation does not play a part in the HC phenotype. In contrast, the *psdR1* mutation, when paired with the $\Delta lasR$ mutation, did not support growth to levels

Figure 3 Growth and proteolysis in pure culture. **(a)** Growth in caseinate medium measured at 12 and 24 h, expressed as CFUs per ml. Means and s.e.m. are shown ($n=3$) and in some cases error bars are too small to be seen. Starting CFUs per ml are statistically the same (one-way analysis of variance (ANOVA), Tukey's multiple comparisons test, $\alpha=0.05$). **(b)** Caseinolytic activity of cultures grown in CAA medium for 12 h, as measured by FITC-casein assay. Caseinolytic activity is shown per OD₆₀₀ to correct for slight variations in the final culture densities in CAA medium. Means and s.e.m. are shown ($n=3$). *Significant differences as determined by one-way ANOVA, Tukey's multiple comparisons test, $\alpha=0.05$. Results of similar magnitude are grouped for clarity.

beyond the *ΔlasR* single mutant, strengthening the role of the *lasR5* allele in the HC phenotype. Taken together, these results show that inactivation of *psdR* increases absolute fitness, and that this effect can compensate for the reduced level of cooperation from partial loss of function in *lasR5*.

Exoprotease activity

To correlate the absolute fitness of each strain with its exoprotease activity, we quantified caseinolysis of culture supernatants using a FITC-casein assay (Twining, 1984; Wilder *et al.*, 2011). This method is more precise than the qualitative skim-milk plate assay used previously (Sandoz *et al.*, 2007). In order to uncouple exoprotease activity from its effect on

growth, we replaced caseinate in our growth medium with CAA, a C-source that does not require QS-dependent proteolysis. All strains harboring the *lasR5* allele showed intermediate levels of extracellular caseinolysis at half the levels of the wild-type *lasR* alleles and roughly three times higher than the *ΔlasR* alleles (Figure 3b). Strains containing *psdR* mutations did not show elevated caseinolysis compared with the wild-type. These results confirm that *lasR5* is a partial loss-of-function mutation, and further show that *psdR* has no effect on QS-dependent exoprotease production, consistent with its role in regulating intracellular dipeptide metabolism.

Transcriptional regulation of dipeptide transport and processing

We have provided evidence that *psdR* mutations are explicitly linked to significant increases in the fitness of *P. aeruginosa* in a cooperative environment. As indicated above, PsdR is a transcriptional repressor of several neighboring genes involved in the transport and processing of dipeptides in *P. aeruginosa* (Kiely *et al.*, 2008). Specifically, PsdR represses transcription of *mdpA*, which codes for the cytoplasmic dipeptidase MdpA, as well as *dppA3*, the first gene in a dipeptide transport gene cluster annotated *dpp* for a homologous region in the *Escherichia coli* K12 genome (Kiely *et al.*, 2008). Associated with this gene cluster is a gene coding for the porin OpdP that is implicated in the uptake of single amino acids as well as dipeptides in *P. aeruginosa* (Tamber and Hancock, 2006).

Interestingly, our previous transcriptome analysis of *P. aeruginosa* grown in rich medium indicated that *mdpA* expression was affected by *rhl*-QS. Addition of the LasI-generated signal to a signal synthesis mutant only induced expression by 1.6-fold, but addition of both acyl-HSL signals induced expression by ninefold (Schuster *et al.*, 2003). Thus, it was conceivable that *lasR* affected dipeptide transport and processing mainly indirectly, through its effect on the *rhl* system, although this regulation is nutritionally conditional (Medina *et al.*, 2003; Dekimpe and Deziel, 2009; Mellbye and Schuster, 2014). To investigate a possible link between QS and dipeptide metabolism in our experimental system, we quantified the transcript levels of *dppA3* and *mdpA* during growth in CAA medium for our set of eight *P. aeruginosa* strains used in the previous sections. Using quantitative reverse-transcriptase PCR, we assessed transcription in exponential and early stationary phases, corresponding to OD₆₀₀ values of 0.5 and 1.5, respectively. We found that for either gene at any growth phase tested, relative expression could be sorted by *psdR* allele, with at least an order of magnitude separating functional *psdR* alleles from those harboring *psdR1* or *ΔpsdR* (Figure 4). Importantly, none of the *lasR* alleles substantially influenced

Figure 4 Expression of *dppA3* and *mdpA*. Relative transcript levels of *dppA3* (a) and *mdpA* (b), as determined by quantitative reverse-transcriptase PCR (qRT-PCR). Relative expression during exponential (OD₆₀₀ = 0.5, empty bars) and early stationary (OD₆₀₀ = 1.5, filled bars) growth phases in CAA medium are shown. Means and s.e.m. are shown ($n=3$). *Significant differences as determined by one-way analysis of variance (ANOVA), Tukey's multiple comparisons test, $\alpha=0.05$. Results of similar magnitude are grouped for clarity.

expression of *dppA3* or *mdpA* in our experiments. This result indicated that, under the growth conditions employed here, the regulation of dipeptide transport and processing is dependent on *psdR* but independent of *lasR*. Hence, control of the relevant 'private' goods—the cellular dipeptide uptake and processing machinery—occurs independently of QS-mediated 'public' goods in our system.

Relative fitness of evolved and defined strains in co-culture

Next, we measured the relative fitness of our set of strains through pairwise comparisons in co-culture, again employing caseinate medium that requires QS-dependent cooperation. We introduced an

antibiotic resistance marker into one of the two strains at a neutral chromosomal site to allow differentiation in co-culture. The marker itself has no effect on growth (Wilder *et al.*, 2011). With the marked strain at an initial frequency of 0.01 (1%) in starting populations of $\sim 2 \times 10^7$ CFUs per ml total, we allowed competitions to proceed for 24 h, and calculated the relative fitness (w) as the ratio of the average growth rates (Malthusian growth parameters) (Lenski *et al.*, 1991; Wilder *et al.*, 2011).

First, we sought to ensure that selection for *psdR* mutants in the original *in vitro* evolution experiments was not just a general feature of prolonged growth but was tied to the specific growth medium. We therefore initiated defined co-cultures of the Δ *psdR* mutant and the wild-type in different growth media, at a mutant frequency of 0.01. We used a complex medium (MOPS-buffered LB) and M9 minimal medium with essentially fully hydrolyzed casein (CAA) as the sole C-source. The Δ *psdR* mutant did not enrich in either LB + MOPS or CAA media, confirming that adaptive mutation of *psdR* is linked to the cooperative media we employed (Figure 5a). To confirm that the increased absolute fitness of a *psdR* mutant can be attributed in part to increased uptake and metabolism of dipeptides, we also employed M9-minimal medium with the dipeptide GlyGlu as the sole carbon source (Kiely *et al.*, 2008). The Δ *psdR* mutant exhibited a high degree of relative fitness in this medium very similar to the *psdR1* mutant in caseinate medium (Figure 5a and first column of Figure 5b), indicating that dipeptide uptake and metabolism is indeed a target of selection in our cooperative growth environment.

Second, we sought to compare the relative fitness of mutant alleles (initial frequency of 0.01) against the wild-type ancestor to better understand the population dynamics at the beginning of our previous *in vitro* evolution experiment. The *lasR5* mutant modestly enriched in wild-type co-culture, as did the Δ *lasR* mutant in accordance with previous studies (Figure 5b) (Sandoz *et al.*, 2007; Wilder *et al.*, 2011). This result was expected as an individual that decreases investment in a secreted 'public good' while still taking advantage of its production by cooperators should exhibit higher relative fitness (West *et al.*, 2006). A *psdR1* mutant had a tremendous relative fitness advantage with respect to the wild-type, consistent with its high absolute fitness, and mirroring the early dominance of *psdR* mutants during *in vitro* evolution (Figure 5b). When we combined either of the *lasR* mutant alleles with *psdR1*, the average relative fitness again was well above that of the *lasR* mutants alone, demonstrating the independence of *psdR* fitness from LasR regulation (Figure 5b).

We then aimed to understand the relative fitness dynamics after the emergence and dominance of *psdR* mutations in the evolved populations. This time we initiated competitions with the *psdR1*-defined mutant in majority (initial frequency of

Figure 5 Relative fitness. (a) Relative fitness of a $\Delta psdR$ mutant in co-culture with its wild-type parent in rich and defined media, initiated at a mutant frequency of 0.01. LB, LB + MOPS; CAA, M9-CAA; GlyGlu, M9-GlyGlu. (b) Relative fitness of defined mutants in caseinate co-culture. Pairs of the respective rare and abundant strain were initiated at a ratio of 1:99. Relative fitness values were calculated as the ratio of Malthusian growth parameters (w) after 24 h, with the exception that experiments in M9-GlyGlu were allowed to proceed for 7 days to allow the co-cultures to reach saturation. Values of w signify whether the rare strains grow faster ($w > 1$) or grow slower ($w < 1$) than the respective abundant strains. Bars represent means ($n = 3$), and means are significantly different from $w = 1$ (one sample t -test, $P < 0.05$), unless designated by §. NS, difference between two conditions not significant (unpaired t -test, $P > 0.05$). Difference in mean relative fitness of $\Delta psdR$ in M9-GlyGlu co-culture with WT (a) and $psdR1$ in caseinate co-culture with WT (b) is not significant (unpaired t -test, $P = 0.23$).

0.99). Both $psdR1$ $\Delta lasR$ and $psdR1$ $\Delta lasR$ double mutants displayed relative fitness of > 1.0 , as would be required for their enrichment in the original *in vitro* evolution experiments (Figure 5b). The difference in the relative fitness between the two strains is reflected in their relative abundances during *in vitro* evolution (Figure 2a). Interestingly, a defined mutant with the $\Delta lasR$ allele alone was not able to enrich against the defined $psdR1$ mutant, further demonstrating the effect that a large increase in absolute fitness can have on relative fitness against an obligate cheater.

Fourth, to investigate resistance of the HC to obligate cheating, we initiated competitions with the HC genotype ($psdR1$ $\Delta lasR$) in majority (initial frequency of 0.99). We observed resistance to invasion by the $\Delta lasR$ cheater, but when the obligate cheater allele is paired with the evolved $psdR$ allele, the $psdR1$ $\Delta lasR$ relative fitness again rose above 1.0 (Figure 5b).

Taken together, we confirmed our original predictions of the evolutionary trajectories (Figure 2g) of an evolving *P. aeruginosa* population. However, these relative fitness measurements do not fully explain the sustained cooperative growth of the evolved population. While even the $psdR$ mutant was susceptible to subsequent invasion by $psdR1$ $\Delta lasR$ mutants, it is plausible that it would tolerate a higher proportion of cheaters, thereby maintaining high population growth in cooperative growth environments.

Cheater load

To finally determine the effect of increasing fractions of cheaters on the mean group fitness of the entire population, or cheater load, we again used defined co-culture experiments in caseinate medium. We varied the initial frequencies of an obligate

Figure 6 Cheater load. Cheater load expressed as relative growth yield of the entire population. Co-cultures of a $\Delta lasR$ mutant cheater and its wild-type parent (filled bars), and of a $psdR1$ $\Delta lasR$ double mutant cheater and its $psdR1$ single mutant parent (empty bars) were grown for 24 h in caseinate medium. Starting cheater frequencies are indicated on the horizontal axis. Growth yield of each parent strain culture without cheater is set to 100%. Means and s.e.m. are shown ($n = 3$). *Significant difference from respective parent strain without cheater as determined by unpaired t -test ($P < 0.05$).

cheater, $\Delta lasR$ or $psdR1$ $\Delta lasR$, with respect to the cooperating parent strain, wild-type or $psdR1$, respectively, and quantified total population growth after 24 h. As expected, we found the burden of cheaters was significantly lower for the $psdR1$ mutant compared with the wild-type. Significant decreases in population productivity in the $psdR1$ background did not occur until the cheater was at a frequency of ≥ 0.75 , compared with 0.25 for the wild-type (Figure 6). This result demonstrated that $psdR1$ mutation helps stabilize cooperative, proteolytic growth as long as obligate cheaters are not the majority.

Our *in vitro* evolution experiments were initiated with pure cultures of wild-type bacteria. Under these conditions, there was essentially no adaptive race between cooperators and cheaters initially, because the non-social adaptation emerged first and quickly dominated the population. Of course, these mutants with non-social adaptations were then subject to invasion by cheaters that also carry the adaptation. It is possible that an adaptive race between these two evolved genotypes would eventually result in a second non-social mutation that further sustained cooperative growth, consistent with previous work on long-term microbial adaptation (Wiser *et al.*, 2013). This stochastic scenario may explain why we observed a collapsing population in only one out of five *in vitro* evolution experiments (Figures 2b–f). Genome sequencing of late isolates beyond day 12 would be required to confirm this notion. As in the study by Waite and Shou (2012), we found that in defined co-cultures, non-social adaptation conferred resistance to cheaters with an otherwise wild-type background. However, in contrast to Waite and Shou (2012), evolved cheaters were still able to invade their cooperating parent strains. Our result is plausible in that cooperators, no matter how evolved, inevitably divert a portion of their resources into the secretion of public goods, resulting in an

The high number of independent *psdR* mutations during *in vitro* evolution was a surprise, raising the possibility that this locus is a mutational hot spot.

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However, this notion is not supported by the analysis of published *P. aeruginosa* genomes. Out of 18 genomes in the NCBI (National Center for Biotechnology Information) database, 17 contain a *psdR* homolog with $\geq 99\%$ identity, and 9 of which show 100% identity (Altschul *et al.*, 1990) (<http://blast.ncbi.nlm.nih.gov/>). This ubiquity and sequence conservation implies that a functional PsdR is likely necessary for the evolutionary success of *P. aeruginosa* in its natural environment, although the situation may be different for other *Pseudomonas* species with *dpp* operons. *P. protegens* Pf-5 (formerly *P. fluorescens*) contains a truncated, presumably inactive *psdR* allele, whereas several other *Pseudomonas* spp. do not carry *psdR* at all (Kiely *et al.*, 2008). The maintenance of a functional PsdR in natural *P. aeruginosa* isolates suggests that proteolysis may limit growth more often than subsequent peptide processing, or that PsdR activity may be modulated through a natural ligand and derepression would sufficiently increase dipeptide metabolism. PsdR is a Mer-type regulator with a helix-turn-helix DNA-binding domain and a cupin sensor domain that has the potential to respond to a variety of environmental stimuli (Brown *et al.*, 2003; Kiely *et al.*, 2008).

A second, beneficial effect of the *psdR* mutation was that it was able to promote cooperative growth near wild-type levels when paired with a partial loss-of-function *lasR* allele, *lasR5*. This mutation in *lasR*, by itself, conferred intermediate levels of proteolysis and proteolytic growth in culture. Thus, the random emergence of certain *lasR* mutations, particularly in an adapted parent, is not detrimental to cooperative growth. Although *lasR5* affects LasR-dependent phenotypes other than caseinolysis (Sandoz *et al.*, 2007), the precise impact on the entire regulon is not clear. The *lasR5* mutation substitutes a valine for a nonconserved alanine at position 228 in the DNA-binding domain of LasR, presumably weakening, but not completely eliminating, interaction with target promoters.

Given the properties of the *lasR5* mutation, one might expect a HC to exhibit lower relative fitness than a fully *lasR*-deficient cheater when paired with a cooperator. We observed this difference with strains harboring the *psdR1* mutation, but not with those harboring functional PsdR. A possible explanation for this discrepancy could be the large size of the *lasR*-controlled QS regulon and the difference in the relative burdens it imposes on cooperators with and without the *psdR* mutation. Potential fitness differences between *lasR5* and $\Delta lasR$ alleles stemming from the variable costs of cooperative extracellular proteolysis (and other LasR-dependent behaviors) could be effectively masked by PsdR-mediated repression of dipeptide uptake and processing, and only manifest after this rate-limiting step has been removed through mutation of *psdR*. Thus, genetic context may be important when considering the relative fitness contributions from a cooperative

allele. This idea is also supported when interpreting our results in the framework of kin selection theory.

Kin selection theory, encapsulated in Hamilton's rule, states that cooperation evolves if $rb - c > 0$, where b is the benefit of cooperation, c is the cost of cooperation and r is the genetic relatedness between actors and recipients (Hamilton, 1964a,b). It has been shown that the cost c of bacterial cooperation may decrease with increased resource supply (Brockhurst *et al.*, 2008). Analogously, *psdR* mutation appears to alleviate c by increased use of the products of protease digestion. This reduction in c does not require a direct mechanism, that is, a direct effect of *psdR* on the cooperative trait itself, but merely reflects the context in which the behavior is performed. Given the nonlinearity of fitness effects in our system (including a synergistic effect from QS induction and a saturating effect from protease secretion), further analysis of frequency-dependent relative fitness in the context of a generalized form of Hamilton's rule would be required to precisely quantify b and c (Smith *et al.*, 2010).

More broadly, cycles of non-social, genetic adaptation and cheating are unlikely to maintain cooperative behavior in the long term as environmental adaptation is expected to eventually reach an optimum. Non-social adaptation through mutation likely works in concert with other mechanisms that stabilize cooperative behavior, and may be particularly beneficial early in the evolution of cooperative behavior. The generally high phenotypic plasticity of present-day microbes with unpredictable life histories would appear sufficient for coping with most changes in their natural environment. In microbes, other stabilizing mechanisms include positive assortment of cooperating individuals through, for example, colonial growth (Fletcher and Doebeli, 2009), the linkage of cooperative behaviors with other essential traits through pleiotropy (Foster *et al.*, 2004) and metabolically prudent regulation of public goods such that their production is only initiated if the limiting nutrient is not also a building block of the good (Xavier *et al.*, 2011; Mellbye and Schuster, 2014). It seems that pleiotropic control of extracellular proteolysis and subsequent intracellular metabolism via QS would be a reasonable strategy to curtail cheating, and a recent investigation using a similar *in vitro* evolution system has provided support for this notion (Dandekar *et al.*, 2012). QS cheaters that do not contribute to proteolysis would be punished with reduced nutrient uptake and processing. We found that *lasR* indeed controls *mdpA* transcription in rich medium (Schuster *et al.*, 2003), but not in the minimal medium used in this study. Of course, the relative fitness advantage of *lasR* mutant cheaters is consistent with the latter. Even if pleiotropic control played a role here, our results suggest that QS regulation of *mdpA* or related genes would be subject to strong counterselection whenever dipeptide uptake and processing was growth-rate limiting.

In summary, we have shown that non-social, genetic adaptation to a new growth environment that requires QS can help maintain cooperative behavior in *P. aeruginosa* populations. The adapted population is still vulnerable to invasion by cheaters that also carry the adaptation. However, a higher intrinsic growth rate affords higher tolerance to these cheaters, and some *lasR* mutations even contribute to cooperative growth.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Conceived and designed the experiments: KLA, JW and MS; performed the experiments: KLA and JW; analyzed the data: KLA; contributed reagents/materials/analysis tools: KLA, JW, KG and MS; wrote the paper: KLA and MS.

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