Analysis of Mutations Affecting Cooperation in *Pseudomonas aeruginosa* Populations

AN ABSTRACT OF THE THESIS OF

Tanner M. Robinson for the degree of Master of Science in Microbiology presented on August 30, 2018.

Title: Analysis of Mutations Affecting Cooperation in *Pseudomonas aeruginosa* Populations.

Abstract approved: _____

Martin Schuster

In a process called quorum sensing (QS), the bacterial pathogen *Pseudomonas aeruginosa* uses small diffusible signals to coordinate cooperative behaviors via secreted "public goods". Under QS-dependent growth conditions, social cheaters arise with mutations in *lasR*, the gene for the primary QS signal receptor. These cheaters do not produce public goods. They impart a burden on the cooperators in the population that can lead to a tragedy of the commons in which the population collapses. During *in vitro* evolution on casein medium that requires QS-dependent protease secretion, a non-social adaptation occurs early in the transcriptional repressor gene *psdR* that increases peptide uptake, allowing toleration of a greater cheater load and delaying population collapse. In this study, we use whole genome sequencing to identify mutations that

arise late in experimental evolution. We characterize prevalent loss-of-function mutations in the alternative sigma factor gene *rpoS*. These mutants display normal QS abilities in non-cooperative growth conditions, but are unable to grow in single culture on casein. *rpoS* mutants show increased fitness when grown in co-culture with the wild-type. Previous microarray data show that RpoS represses the same set of peptide uptake genes as PsdR and induces a secreted aminopeptidase, PaAP, providing a possible mechanism. Overall, our results suggest that inactivation of *rpoS* results in both cooperative and non-cooperative adaptations in *P. aeruginosa*, contributing to our understanding of processes that shape cooperative behavior in bacteria.

Analysis of Mutations Affecting Cooperation in *Pseudomonas aeruginosa* Populations

by
Tanner M. Robinson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented August 30, 2018

Commencement June 2019

Master of Science thesis of Tanner M. Robinson presented on August 30, 2018.

APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Tanner M. Robinson, Author

ACKNOWLEDGEMENTS

The author expresses sincere appreciation to Dr. Martin Schuster for his continual guidance and support throughout this project. He also would like to thank his fellow lab members, current and past, for helping in various stages of the work. Finally a special thanks goes to his family for their support and encouragement.

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Introduction

Bacteria communicate through a process called quorum sensing (QS) (1, 2). In gram-negative bacteria, members of a population of cells synthesize and secrete diffusible autoinducers (AI) called acyl homoserine-lactones (AHL) (1-3). As cell density rises, the concentration of AHL increases, reaching a critical quorum threshold (1-3). At this threshold, the concentration of AHL is high enough to bind intracellular receptors which activate (or suppress) transcription of an array of genes. This communication allows a coordinated regulation of "public goods" which are used by the community as a whole. These secreted goods include exoenzymes involved in nutrient acquisition, antibiotics and toxins involved in microbial competition, exopolysaccharides involved in biofilm formation, and siderophores involved in iron scavenging, among others (1, 3-6). QS can thus allow survival in otherwise inaccessible environments (6). *Pseudomonas aeruginosa* is a gram negative opportunistic human pathogen particularly useful for studying QS systems. Several QS circuits are present in this bacterium, but are principally controlled in a hierarchical manner by the LasI-LasR acyl-HSL system (7). LasI is an autoinducer synthase which produces *N*-3-oxododecanoyl-homoserine lactone (3O12C-HSL) as an AI (7). LasR is the cognate receptor for 3O12C-HSL that acts as a transcription factor when bound, turning on QS (7). Activation of other QS circuits in *P.*

aeruginosa, particularly the RhlI-RhlR acyl HSL system, are among the downstream effects of this regulator (7, 8). Most notably, *P. aeruginosa* QS activates secreted proteases involved in the digestion and utilization of extracellular protein (9, 10).

This process has served as a model to study the evolution and maintenance of microbial cooperative behavior (3, 11). The evolutionary stability of cooperation is threatened by social cheating (12). Because production of secreted public goods is costly, cheaters benefit from the cooperative behavior by others without contributing themselves (13-15). These cheaters gain a fitness advantage, and thereby increase in frequency quickly within a population (14-16). When cheater levels become too high for the cooperators to support, social evolution theory predicts the population will collapse due to a 'tragedy of the commons' (12, 17).

In *P. aeruginosa* these population dynamics can be observed when grown under conditions that require QS, specifically in a minimal medium with casein as the sole carbon source. Extra-cellular proteases required to break down casein into products usable by the cell are regulated by QS in *P. aeruginosa* (9, 18). Our previous *in vitro* evolution experiments under these conditions revealed the emergence of *lasR* mutant cheaters that were unable to produce extracellular proteases themselves but were able to utilize the breakdown products from the proteases produced by wild-type cells (13, 14). However, unlike predicted by social evolution theory and recapitulated in defined co-cultures, *lasR* mutants generally did not exceed 30% of the population and did not cause population collapse (14-16). In search of a mechanism that could explain this

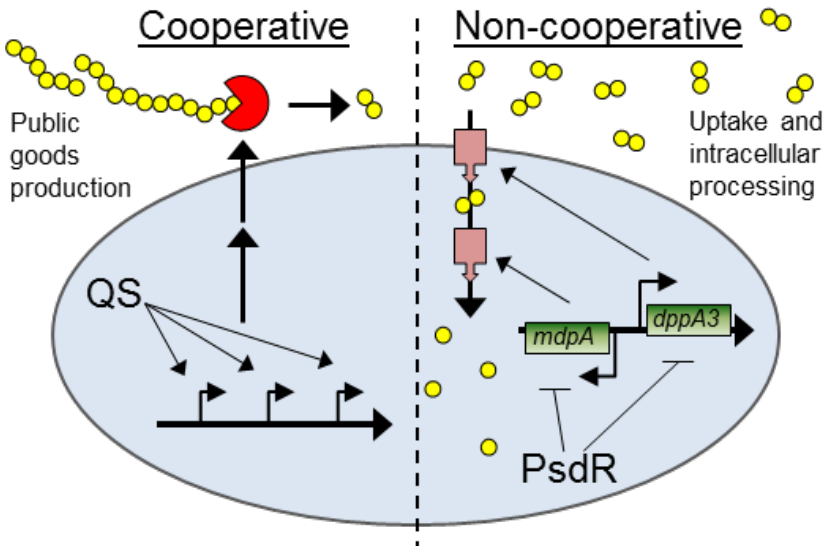

Figure 1 Summary of the cooperative and non-cooperative mechanisms leading to increased growth rate and increased tolerance of cheaters. Extracellular proteases (red) controlled by cooperative QS degrade polypeptides (yellow chains, each circle represents one amino acid) outside the cell. The non-cooperative QS independent system is involved in the transport and processing of dipeptides. Genes (green) code for proteins (pink) that do the actual transport (DppA3) and intracellular breakdown (MdpA) of dipeptides. A mutated PsdR leads to derepression of the non-cooperative system. Adapted from ref. 13.

phenomenon, we identified a mutation in the regulatory gene *psdR* that helped to stabilize the population in the presence of cheaters (15). PsdR is a transcriptional repressor of genes involved in amino acid and dipeptide uptake and metabolism (15, 19). When mutated, *mdpA* and *dppA3* are among the derepressed genes. The former codes for the cytoplasmic dipeptidase MdpA while the latter is the first gene of a gene cluster involved in dipeptide transport into the cell (19). PsdR mutations increase both the absolute and relative fitness of those members of the population that harbor it (15). Cooperators are still susceptible to invasion by cheaters with the *psdR lasR* double mutant genotype, but are better able to bear the load and resist take over by cheaters due to increased growth rate (15). Thus, the casein is digested initially in a QS-

dependent manner by secreted proteases, with a QS-independent adaptation allowing for better utilization of the breakdown products (Figure 1) (15).

While the effect of a nonfunctional PsdR is significant enough to promote cooperation during *in vitro* evolution, it is nonetheless unable to fully stabilize cooperation in defined co-culture (14-16). Cheaters still invade and disrupt cooperative growth above a certain threshold (15). We hypothesized that at least one additional mutation must have occurred to further stabilize the system, averting a tragedy of the commons. To identify and characterize this mutation we employed whole-genome sequencing, genetic analysis, protein modelling, and growth experiments. We found a common mutation in the *rpoS* gene, encoding an alternative sigma factor associated with stationary phase and the stringent response (20, 21), in about half of the sequenced cooperators. Deficiency in *rpoS* provides an additional fitness benefit relative to single *psdR* mutants. We show that *rpoS* mutants, while not deficient in QS, are unable to grow in single culture but thrive when rare in co-culture similar to *lasR* mutants. However, in addition to this obligate, social phenotype, *rpoS* mutants also show non-social adaptation to the growth environment similar to *psdR* mutants. We propose that this complex behavior may further delay population collapse under cooperation-dependent conditions.

Materials and methods

Strains and culture conditions

For all experiments, we used the *P. aeruginosa* PAO1 wild-type (WT) or mutant derivatives. For a complete list of strains, see Table 1. Unless otherwise specified, strains were grown in Lennox lysogeny broth buffered with 50 mM 3-(*N*-morpholino)-propanesulfonic acid (LB-MOPS), pH 7.0. All cultures were incubated at 37°C with shaking at 250 rpm.

For whole-genome sequencing, isolates were chosen from *in-vitro* evolution experiments according to QS-dependent phenotypes (14-16). The PAO *rpoS5* and PAO *psdR1 rpoS5* mutants were constructed using a two-step allelic exchange as described (22). The *rpoS* region was PCR-amplified with the desired evolved frameshift mutation using primers 5'- NNNNNNAAGCTTAGGTCGTCGATCGCAACGGTTC-3' and 5'-NNNNNNTCTAGACGTCACTCGACAGGCCATTCTTCTC-3' containing flanking HindIII and XbaI restriction sites (underlined), respectively. The PCR fragment was cloned into *Xba*I and *Hin*dIII-digested pEX18Tc for use in allelic exchange (22). The resulting construct was introduced into either the PAO1 WT or the previously constructed PAO *psdR1* mutant by allelic exchange by conjugation with a suicide plasmid (22). Electroporation was used to construct PAO *psdR1 rpoS*::Gm^R based on the procedure previously described (23). Briefly, 5 mL of overnight culture in LB Lennox broth were aliquoted equally into four microcentrifuge tubes. After centrifuging for 1 min at 16,000 × *g* the pellets were washed two times in 1 mL room temperature 300 mM sucrose. Cells were

suspended at 100 µL total volume in 300 mM sucrose. 500 ng of purified PAO *rpoS*::Gm^R chromosomal DNA was mixed with 100 µL electrocompetent PAO *psdR1* cells and transferred to a 2 mm gap electroporation tube. A pulse (25 µF, 200 Ω, 2.5 kV) was applied and then cells were suspended in 1 mL of LB, transferred to a microcentrifuge tube and incubated for 2 h at 37°C with shaking. 900 µL supernatant was discarded and cells were resuspended in the remaining liquid before being spread onto LB plates containing gentamicin (50 µg/mL).

Table 1 Bacterial strains and plasmids

Strains	**Relevant Properties**	**Reference or Origin**
Pseudomonas aeruginosa		
PAO1	Wild-type (obtained from M Vasil and U Ochsner)	(24)
PAO Δ*lasR*	PAO1 derivative; Δ*lasR*, unmarked in-frame deletion from amino acids 102–216	(16)
PAO Δ*lasR,* Tp^R	PAO1 derivative; Δ*lasR*, unmarked in-frame deletion from amino acids 102–216; chromosomal miniTn7-Tp^R insertion near *glmS* gene	(16)
PAO *rpoS5*	PAO1 derivative; *rpoS5,* unmarked evolved *rpoS5* allele	This study
PAO *rpoS*::Gm^R	PAO1 derivative; *rpoS*::Gm^R, marked mutant with *aacC1* inserted in the *rpoS* gene	(9)
PAO *psdR1*	PAO1 derivative; *psdR1*, unmarked mutant in which wild-type *psdR* was replaced with *psdR1*	(15)
PAO *psdR1 rpoS5*	PAO *psdR1* derivative; unmarked double mutant harbors the *psdR1* and *rpoS5* mutations	This study
PAO *psdR1 rpoS*::Gm^R	PAO *psdR1* derivative; marked double mutant containing both *psdR1* and *rpoS*::Gm^R mutations	This study
Escherichia coli		
DH5α	F- Φ80*lacZYA-argF* U169 *recA1 hsdR17* (r^-_k , m^+_k) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
SM10	*thi thr leu tonA lacY supE recA:*:RP4-2-Tc::Mu Km^R λ*pir*	(25)
Plasmids		
pEX18Tc	Conjugative suicide plasmid; Tc^R	(22)
pEX18Tc *rpoS*5	pEX18Tc with frameshift *rpoS5* allele at residue 20	This study

Isolate phenotype assays

To confirm previously recorded QS-dependent phenotypes of each *in vitro*-evolved *P. aeruginosa* isolate, we conducted skim-milk proteolysis, adenosine growth, and pyocyanin production assays (14). A single freshly streaked colony from each isolate was patched onto skim milk plates (4 % skim milk in ¼-strength LB) and adenosine agar plates (M9 minimal medium with 0.1 % adenosine as the sole carbon source) and incubated at 37°C. After 18 h, skim milk proteolysis was evaluated as a zone of clearance surrounding each colony. After 48 h, growth was evaluated on adenosine plates. We qualitatively evaluated pyocyanin production as blue-green pigmentation of overnight LB broth cultures inoculated with a freshly-streaked colony. For all assays, the respective phenotype was compared to a positive WT control and a negative *lasR* mutant control.

DNA preparation and sequencing

Chromosomal DNA was extracted from 30 *in vitro*-evolved isolates and the PAO1 parent strain using the Gentra Puregene Yeast/Bacteria Kit from Qiagen and suspended in 100 µL ddH_2O. Whole-genome sequencing was done using Illumina HiSeq 3000 by the Center for Genome Research and Bioinformatics (CGRB) at Oregon State University, with 150 bp paired end reads. This produced between 8,665,795 and 15,100,212 reads per sample (median 12,497,204). Low quality reads and primer sequences were trimmed using Trimmomatic (version: 0.36) (26). Adapter sequences and 10 bases leading or trailing were cut off and reads with a phred score below 20 in a 4 base sliding

window or less than 40 bases were discarded (26). Reads were then aligned to the reference and genomes assembled and manipulated with Bowtie2 (version: 2.3.2) and SAMtools (version: 1.3), using the WT parent as a reference for each isolate genome (27, 28). The PAO1 parent strain reads were aligned to the online reference *P. aeruginosa* PAO1 from www.pseudomonas.com and isolate reads were aligned to the parent (26, 29). Mutations were called using a Bayesian statistical method, Freebayes (version: v0.9.21-15-g8a06a0b) (30). Assembled genomes and mutations were visualized using Geneious (31). Targeted Sanger-sequencing of *rpoS* was also conducted on DNA from twenty day 12 isolates at the CGRB using the following primers flanking the gene: 5'-GCTTGAGTCGAACTCATGCAAG-3' and 5'-CGGCATTTATCTACTTAGGCTCA-3'.

Absolute and relative fitness assays

A single colony from freshly streaked plates was inoculated in 4 mL LB-MOPS medium and incubated for 18 h. Four mL of M9 minimal medium containing 1% (w/v) casein as the sole carbon source were inoculated at an optical density at 600 nm (OD_{600}) of 0.05 and incubated for 24 h. Colony-forming units per mL (CFU/mL) were determined at 0, 6, 12, and 24 h by serial dilution and spot plating.

For co-cultures, single freshly streaked colonies were inoculated in 4 mL LB-MOPS medium and incubated for 18 h. OD_{600} was quantified to formulate strain mixtures at a ratio of 99:1. To distinguish strains in co-culture, the rare strain carried a chromosomal antibiotic resistance marker (either gentamicin, tetracycline, or trimethoprim). Six hundred µL of casein-M9 minimal medium in 96 deep well blocks

were inoculated with the co-cultures at a starting OD_{600} of 0.02. Co-cultures were incubated for 24 hours, and CFU/mL was determined at 0, 12, and 24 h. Strain ratios were quantified by plating on LB plates with no antibiotic, and on LB plates containing gentamicin (50 µg/mL), tetracycline (100 µg/mL), or trimethoprim (100 µg/mL). CFU/mL was calculated as listed before. The relative fitness *w* of the rare strain was calculated as the ratio of Malthusian parameters: $w = \frac{\left(\frac{(lnN1t)}{(lnN10)}\right)}{\frac{(lnN2t)}{(lnN20)}}$, with N_{1t} as the final CFU/mL of the rare strain and N_{1o} as the initial CFU/mL. N_{2t} and N_{2o} are the final and initial cell density of the abundant strain, respectively. Statistically significant differences in relative fitness values in comparison to each other were determined by a two-sample t-test (equal variance; $\alpha = 0.05$). Statistically significant differences compared to no change ($w = 1$) were determined by one sample t-test ($\alpha = 0.05$).

Casein proteolysis assay

A single freshly streaked colony was used to inoculate 4 mL of LB-MOPS and incubated for 24 h. Cultures were centrifuged and supernatant liquid was filtered (0.2 µm pore size). Wells cut of skim-milk agar plates with the back side of a Pasteur pipet were filled with 200 µL of each culture supernatant. Agar plates were incubated for 24 hours at 37°C. The diameter of each circular zone of proteolysis was measured. Statistically different zone measurements were determined by two-sample t-test as above.

Gly-Glu dipeptide growth

To evaluate the ability of each strain to grow with a dipeptide as the sole carbon source, growth assays were performed as described previously (19). Strains were pre-cultured according to a previous study (19) in 0.5 % (w/v) glucose M9 minimal medium for 18 h, washed in 1 x M9 salts, and inoculated at an OD_{600} of 0.02 in 4 mL of M9 minimal medium containing 10 mM Gly-Glu dipeptide as the sole carbon source. Incubation at 37°C was allowed to continue until stationary phase was reached for all strains, with OD_{600} measurements taken every 8 or 12 h.

Results

rpoS mutants arise in cooperator populations

Previous work in our laboratory identified *psdR* mutation as an integral component in delaying population collapse during long-term cooperative growth of *P. aeruginosa* PAO1 on caseinate medium (15). However, this mutation is not sufficient by itself to prevent population collapse. We therefore hypothesized that another mutation contributes to the stabilization of cooperative growth. To test our hypothesis, we sequenced the genomes of 30 isolates from our two previous *in vitro* evolution experiments (14, 16). We chose 30 candidates from the final day of sub-culturing, including all nine available isolates from the earlier Sandoz study and 21 isolates from the more recent Wilder study (14, 16). As determined in these studies, the isolates displayed an array of different QS-dependent social behaviors, distinguishable by two simple phenotypic assays. Cooperators produce QS-dependent extracellular proteases that degrade skim milk, a nucleoside hydrolase that permits growth on adenosine as the sole carbon source, and a characteristic blue-green metabolite, pyocyanin. Phenotypes related to QS ability were recorded using skim milk and adenosine assays as well as pyocyanin production (Table 2). Cooperators are distinguished by positive marks on both skim milk proteolysis and adenosine growth. Cheaters are distinguished by a negative on all phenotypic screens performed. Hybrid cooperators (HC) are positive for skim milk proteolysis but are unable to grow on adenosine, indicating an attenuated QS phenotype. We repeated the skim milk and adenosine assays here and were able to

confirm previous results (Table 2). We also qualitatively evaluated pyocyanin production in cultures as a separate QS-dependent phenotype (18). All but one cooperator strain secreted pyocyanin.

Table 2 Summary of sequenced isolates

Isolate	Replicate	Phenotype			Mutation		
		Skim Milk	Adenosine	Pyocyanin	Locus	DNA	Amino Acid
Cooperators							
TR02	1	+	+	+	*lasR*	C->T	A228V
					rpoS	G->A	R308T
					psdR	Δ512-529	Δ172-177
TR03	2	+	+	+	*psdR*	Δ512-529	Δ172-177
TR04	2	+	+	+	*psdR*	A->C	Y144S
TR05	2	+	+	+	*psdR*	A->C	Y144S
TR11	3	+	+	+	*rpoS*	C->A	E295X
					psdR	T->C	S56P
TR12	3	+	+	+	*rpoS*	C->A	E295X
					psdR	T->C	S56P
TR13	3	+	+	+	*rpoS*	G->A	P210L
					psdR	C->A	Q37K
TR14	3	+	+	+	*psdR*	G->A	G133R
TR15	3	+	+	+	*psdR*	G->A	G133R
TR22	4	+	+	+	*rpoS*	ΔG	Frameshift (20)
					psdR	T->C	F34L
TR23	4	+	+	+	*psdR*	Δ144-147	Frameshift (49)
TR24	4	+	+	+	*pslB*	+CCCGGG	+PG (127)
					psdR	Δ144-147	Frameshift (49)
TR25	4	+	+	+	*rpoS*	+AG	Frameshift (198)
					psdR	A->C	Y144S
TR26	4	+	+	-	*psdR*	+A	Frameshift (126)
Cheaters							
TR06	1	-	-	-	*fleQ*	C->T	R318C
					lasR	G->A	E181K
					psdR	Δ512-529	Δ172-177
TR07	2	-	-	-	*fliR*	ΔG	Frameshift (17)
					PA3330	A->G	S190G
					psdR	Δ512-529	Δ172-177
TR08	2	-	-	-	Upstream *lasR*	+C (-15)	-
					psdR	Δ512-529	Δ172-177
TR16	3	-	-	-	*lasR*	Δ3bp	ΔI 215
					rpoS	C->T	G284S
					psdR	C->A	Y137Stop
TR17	3	-	-	-	*lasR*	C->A	A158E
					psdR	G->A	G133R
TR27	4	-	-	-	*flgI*	C->T	Q105Stop
					lasR	Δ330-342	Δ110-113

Table 2 continued on next page

Table 2 continued

Isolate	Replicate	Phenotype			Mutation		
		Skim Milk	Adenosine	Pyocyanin	Locus	DNA	Amino Acid
Hybrid Cooperators							
TR09	1	+	-	-	*lasR*	C->T	A228V
					psdR	Δ512-529	Δ172-177
TR10	2	+	-	+/-	Intergenic	G->A (3,559,339)	-
					psdR	Δ512-529	Δ172-177
TR18	3	+	-	-	*lasR*	A->G	M212V
					psdR	*	*
TR19	3	+	-	-	PA1194	C->T	V138H
					lasR	A->G	M212V
					psdR	*	*
TR20	3	+	-	+/-	*lasR*	A->G	M212V
					psdR	*	*
TR21	3	+	-	-	*lasR*	A->G	M212V
					psdR	*	*
TR28	4	+	-	+/-	PA2228	C->A	D328Y
					ctpH	G->T	A434S
					rpoS	C->T	M1I
					psdR	+A	Frameshift (126)
TR29	4	+	-	-	*mutL*	Δ1179-1191	Δ393-397
					psdR	+A	Frameshift (126)
TR30	4	+	-	-	*lasR*	A->T	N202I
					PA4037	A->C	E305D
					psdR	ΔCCCT	Frameshift (49)
TR31	4	+	-	-	*lasR*	A->T	N202I
					PA2434	T->C	Q63P
					psdR	ΔCCCT	Frameshift (49)

[1] Numbers indicate replicates from *in vitro* evolution experiments: 1 and 2, ref. 14; 3 and 4, ref. 16.
[2] +, proteolysis positive on skim milk, growth positive on adenosine, pyocyanin production positive; -, no proteolysis on skim milk, no growth on adenosine, no production of pyocyanin; +/-, intermediate pyocyanin production.
* Predicted mutation not identified due to contig gap in genome alignment

Sequencing was performed on the Illumina HiSeq 3000 platform with 150 bp paired-end reads. After trimming low-quality reads and aligning to the parent PAO1 strain as a reference, mutations were called and assessed. Sequencing revealed *psdR* mutations in all but five isolates. Upon closer examination, we found that a contig gap in the assembled genome fell in the middle of the *psdR* gene in four of the five cases.

Due to this, and the discovery of mutations at this locus in every other isolate, we believe each isolate with a contig gap does indeed contain a mutation in this gene. In the cheater genomes, we found a mutation in the *lasR* coding region in five of six isolates. In the remaining isolate, there is an insertion in the -15 position from the *lasR* translation start site that presumably disrupts translation by hampering ribosome binding. HC genomes had mutations in *lasR* and *psdR,* in addition to other loci that were not consistent throughout the group (Table 2). Importantly, in the cooperator genomes, we found a mutation in *rpoS* in 6 out of 14 isolates (43%) (Table 2). An *rpoS* mutation was identified in only one HC and one cheater isolate. This is the only novel mutation consistent within a group, making it a compelling candidate for further study.

The *rpoS* gene encodes an alternative sigma factor of 334 amino acids in length. This sigma factor was originally described in *Escherichia coli* as a master regulator of a

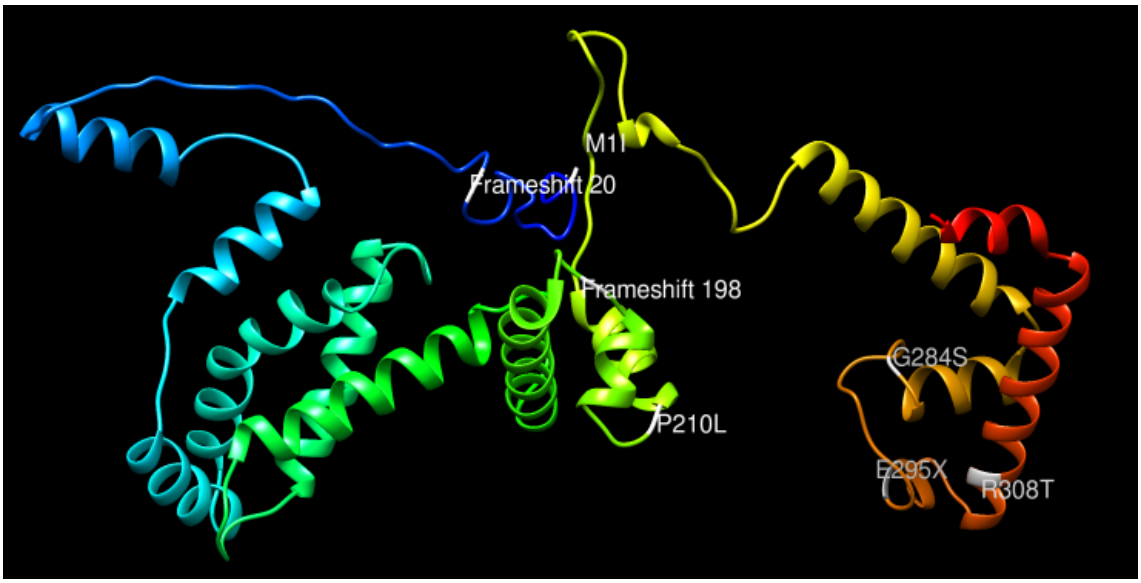

Figure 2 RpoS ribbon diagram produced using I-TASSER with relevant native residues marked in white with the indicated mutation in at least one sequenced isolate. The protein is colored rainbow to represent position with blue at the N-terminus and proceeding to red at the C-terminus.

stress response to nutrient exhaustion in stationary phase (32). In *P. aeruginosa,* it fills a similar role, regulating 772 genes differentially during stationary phase growth (33-35). The various *rpoS* mutations resulted in either an amino acid substitution (*rpoS1*, *rpoS3*, and *rpoS4*), truncation (*rpoS2*), frameshift (*rpoS5* and *rpoS6*), or lost start codon (*rpoS7*) (Table 3). We also mapped each RpoS mutation onto a three-dimensional protein structure (Figure 2), indicating no particular domain preference or clustering. *rpoS1*, *rpoS2*, and *rpoS4* are mutations that either disrupt or delete the DNA binding domain, so we predict they would also disrupt RpoS dependent gene transcription. *rpoS5-7* cause either a non-translated or aberrant protein. As such, we presume each mutation results in a loss of function.

In order to evaluate how early *rpoS* mutations arise in the population we Sanger-sequenced 20 cooperator isolates from day 12 at the *rpoS* locus (16). No *rpoS* mutants were identified among the isolates tested, consistent with the hypothesis that this mutation arises later than *psdR* mutants, providing an additional fitness benefit. We then chose one particular mutation, *rpoS5*, for further analysis. An early frameshift at residue 20 very likely results in a complete loss-of-function protein, thereby simplifying interpretations of mutant phenotypes (Table 3). We cloned the gene harboring *rpoS5* and transferred it into both the WT PAO1 and *psdR1* backgrounds to analyze the effects of this mutation. The *psdR1* mutation results in the deletion of 6 amino acids close to the carboxyl terminus, which has previously been shown to confer a complete loss of function (15).

Table 3 Summary of RpoS mutations

Isolate	Name[1]	Mutation[2]
TR02	*rpoS1*	R308T
TR11	*rpoS2*	E295X
TR12	*rpoS2*	E295X
TR13	*rpoS3*	P210L
TR16	*rpoS4*	G284S
TR22	*rpoS5*	Frameshift (20)
TR25	*rpoS6*	Frameshift (198)
TR28	*rpoS7*	M1I

[1] Designation for the mutation based on which number isolate it was found.
[2] Mutation to the amino acid residues

Enrichment in single culture vs co-culture

To elucidate the importance of the *rpoS* mutation in cooperative behavior, we first evaluated growth in individual culture (absolute fitness) in casein medium. We compared the growth of the *rpoS5* mutant and the *rpoS5 psdR1* double mutant to that of the WT, the *lasR* mutant, and the *psdR* mutant. We also included a previously constructed *rpoS* mutant in which the *rpoS* gene is disrupted by the insertion of a gentamicin resistance cassette (9). This strain grew identical to the *rpoS5* mutant, confirming the notion that the evolved *rpoS* allele confers complete loss-of-function. In contrast to *psdR1*, which shows a marked increase in growth rate compared to the WT, *rpoS5* grew more similar to the *lasR* mutant (Figure 3). Its highest density was reached within 6 hours and plateaued between 6 and 24 hours. Even the *psdR1 rpoS5* double mutant follows this same pattern, indicating a non-functional RpoS is somehow detrimental to cooperative growth in pure culture.

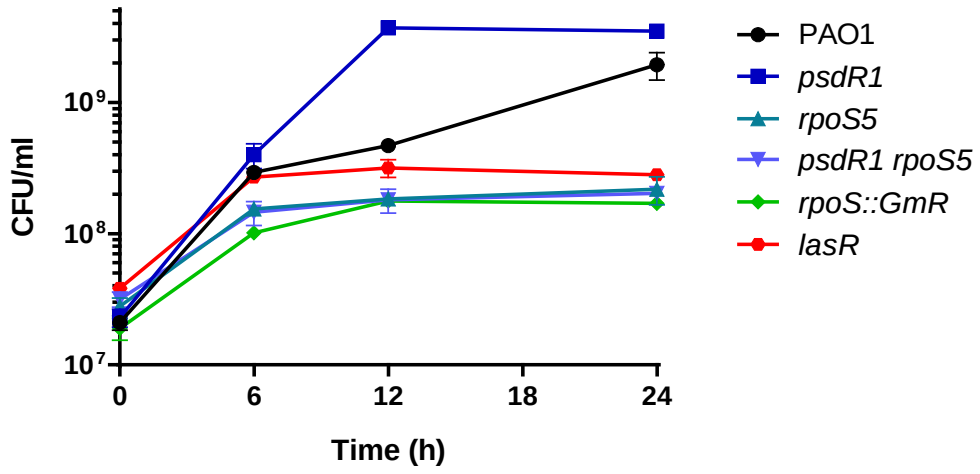

Figure 3 Growth in pure culture. Growth in caseinate medium measured at 0, 6, 12, and 24 h, shown in CFUs per mL. Means are plotted with standard deviation forming the error bars (*n*=3).

Even though *rpoS* mutants showed low absolute fitness, they were nevertheless prevalent in the population during experimental evolution (15, 16), suggesting a high relative fitness in the presence of other cooperating cells. We therefore tested the relative fitness of the *rpoS* mutant and the *rpoS psdR* mutant in co-culture with the WT or the *psdR1* mutant. To distinguish strains in co-culture, we employed *rpoS* mutant strains harboring the gentamicin resistance cassette for subsequent antibiotic selection. When inoculated at 1% initial frequency, *rpoS*::Gm^R and *psdR1 rpoS*::Gm^R show a fitness advantage over WT and *psdR1*, respectively (Figure 4A). In the WT background, this is similar to the *lasR* mutant fitness as measured by the Malthusian parameter. Thus we see *rpoS* mutants do not grow on their own and thrive when rare in the population.

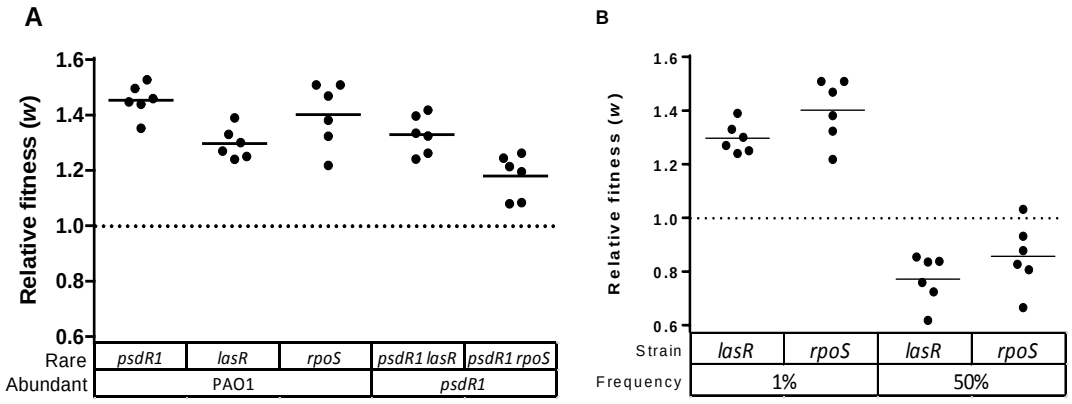

Figure 4 Relative fitness in co-culture. Relative fitness was calculated as the ratio of growth rates (Malthusian parameter) after 24 h in caseinate medium. (A) Relative fitness of the indicated rare strains, inoculated at 1% initial frequency. All means are significantly different from *w*=1.0 as determined by one way t-test (α=0.05). (B) Relative fitness comparison of defined mutants vs WT at two frequencies. Means are plotted as horizontal lines with individual replicates shown (*n*=6). Both 50% frequency means are significantly different from their companion 1% frequency means as determined by two-sample t-test (α=0.05).

Low absolute fitness combined with high relative fitness suggests that *rpoS* mutants, like *lasR* mutants, are cheaters that take advantage of products ("public goods") supplied by others without investing in costly production themselves. A hallmark of cheating is negative frequency-dependence: Cheaters do better when rare in a population because there are more cooperators to exploit (15, 16). We have previously shown that *lasR* mutants exhibit negative frequency-dependent relative fitness when co-cultured with the WT cooperator (15, 16), and we asked whether the same would hold true for the *rpoS* mutant. Indeed, at an initial frequency of 50% the relative fitness drops to near 1, indicating that *rpoS* mutants, like *lasR* mutants, no longer have a growth advantage. This result is consistent with the observation that *rpoS* mutants only comprise a subset of the population and, unlike *psdR* mutants, do not reach fixation (Figure 4B).

Proteolytic activity

Evidence thus far indicates that *rpoS* mutants are non-producing cheaters that may utilize public goods produced by the WT, most obviously secreted enzymes involved in casein proteolysis. We therefore tested the proteolytic capabilities of all our strains to identify differences. To accomplish this, we measured the amount of proteolysis on skim milk agar when incubated with the supernatant of each strain. We used a growth medium (LB) that promoted protease expression but did not require proteolysis for growth (15). In all cases except for the *lasR* supernatant, the clearance zone is not significantly different from the WT (Figure 5). Thus, proteolytic ability does not explain the behavior of the *rpoS* mutant. To obtain additional insights into the mechanism underlying the *rpoS* cheater phenotype, we interrogated our previous microarray data of *rpoS*-dependent genes in *P. aeruginosa* PAO1 (33). The very large size of the RpoS regulon (almost 800 genes) indicates that the behavioral phenotype may be a result of complex pleiotropic regulation. Nevertheless, we identified one candidate gene, *pepB*, that could explain the observed behavior. This gene is highly induced by RpoS and encodes an extracellular aminopeptidase (PaAP, PA2939). PaAP preferentially cleaves leucine from the ends of peptides (36). This activity would not be required for the caseinolytic activity as visualized on skim milk plates, but might still promote growth on casein by making individual amino acids available from break-down products. The extracellular, shared nature of PaAP is consistent with the cheater phenotype of the *rpoS* mutant.

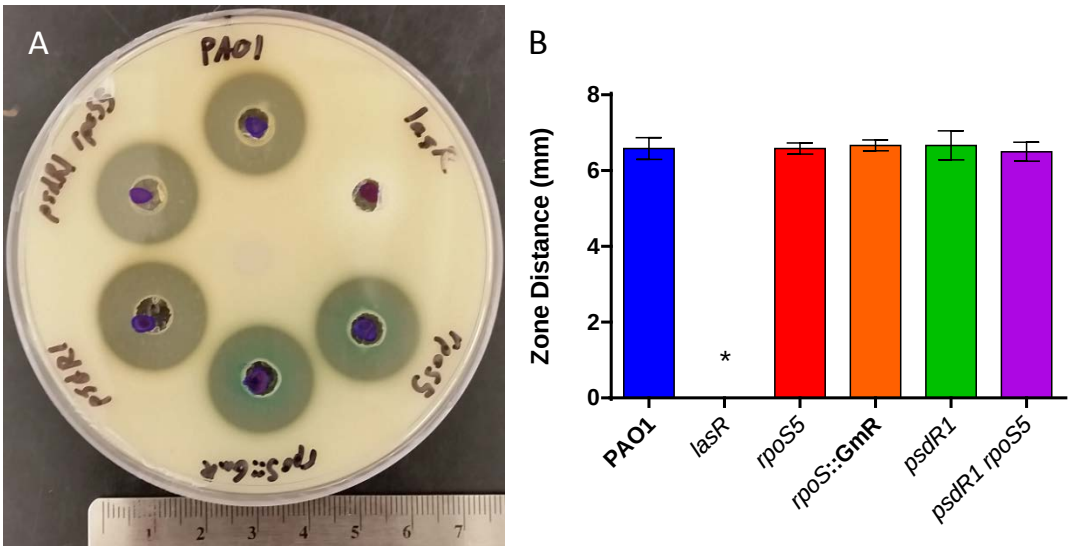

Figure 5 Skim milk proteolysis. (A) Photograph of a representative plate for the assay. Labels of the strain supernatant are listed along the outside of the plate. Blue marks were used as guides for making the wells. The ruler on the bottom is in centimeters. (B) Measurement of proteolysis in the form of zone clearing. Zones were measured from well edge to the edge of the zone. Means are displayed with standard deviation as error bars (*n*=3). * indicates *p*-value < 0.0001 compared to PAO1 with a two sample t-test (α=0.05).

Dipeptide growth

Our previous microarray data (33) further show that RpoS down-regulates (about 2-fold) the expression of the same dipeptide/amino acid uptake and metabolism genes that PsdR suppresses. While the mechanism of action is yet unknown, repression may be indirect via the competition of different sigma factors for RNA polymerase (37). In the absence of RpoS, the RpoD (sigma70) dependent dipeptide/amino acid uptake genes *mdpA* and *dppA3* may be transcribed at an elevated rate. Based on this evidence, we investigated the growth of an *rpoS* mutant (*rpoS5*) on dipeptide medium. When grown with Gly-Glu dipeptide as the only carbon source (15, 19), *rpoS5* indeed conferred a growth advantage compared to its parent, but the *psdR1 rpoS5* double mutant did not

(Figure 6). It is plausible that a combined effect on dipeptide/amino acid uptake is strongest when the evolved *psdR* and *rpoS* alleles each merely confer a partial loss-of-function. Taken together, our findings are consistent with the hypothesis that *rpoS* mutants allow an increase in transcription rates of *mdpA* and the *dpp* gene cluster.

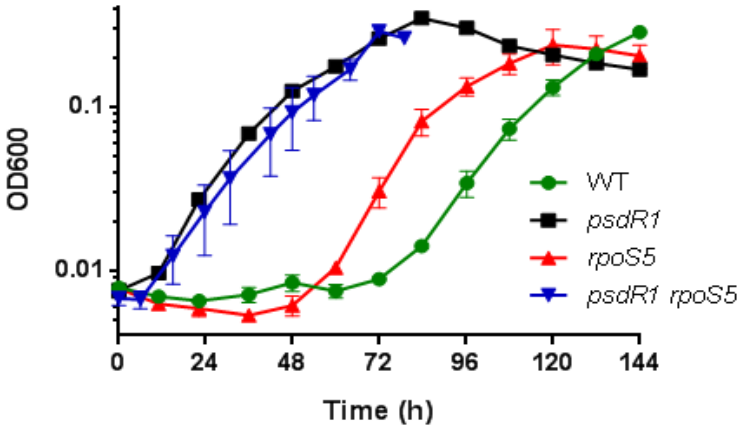

Figure 6 Growth in Gly-Glu dipeptide medium. Combined graph of the growth of WT (green), rpoS5 (red), psdR1 (black), and psdR1 rpoS5 (blue). Means are plotted with standard deviation plotted in error bars (n=5).

Conclusion

In this study we identify a novel player in the experimental evolution of *P. aeruginosa* under conditions that favor QS. Growth in this type of environment selects for both social and non-social adaptations that improve individual fitness to varying degrees. We set out to explain the mechanisms by which a tragedy of the commons is delayed under these conditions. The evolution and maintenance of QS and associated cooperative behaviors seems difficult to explain. This is because cooperation requires non-selfish actions. Kin selection theory, encapsulated in Hamilton's rule, provides an explanation for the evolution of cooperation. This rule states that cooperative behaviors evolve and are maintained when $rb - c > 0$; where *b* is the benefit, *c* is the cost of the behavior, and *r* is the degree of relatedness (12, 38). The costs of QS-dependent public good production by the individual outweigh the benefits. However, in a group of other cooperating cells, an indirect benefit can be realized by helping closely related individuals that also harbor the gene(s) encoding the social behavior. The problem is that selfish individuals in a population can exploit the behavior by not participating in the costly behavior, while still reaping the rewards. This increases their own fitness considerably in conditions that require cooperation in order to survive. Several mechanisms have been identified that curtail cheating and help stabilize cooperative behavior in microbes. These include selfish environmental adaptation of cooperators, as mentioned above, or pleiotropic constraint, such as the co-regulation of private goods with public goods by QS (39). When grown in a medium with both adenosine and

casein as carbon sources, a *P. aeruginosa* population resists invasion by cheaters. Under these conditions, an intracellular enzyme, nucleoside hydrolase (*nuh*) is required to break down the adenosine, while other secreted proteases digest the casein (39). This dynamic of including a private good with the public good makes it difficult for cheaters to gain a fitness advantage (39).

We used a variety of means to investigate the nature of the *rpoS* mutation and why a functional RpoS might be selected against during *in vitro* evolution on casein as the sole carbon source. It is interesting that the mutation did not sweep through the population like the *psdR* mutants, but instead occupies a relatively small percentage of the whole (Table 2). *rpoS* mutants emerged in three out of four replicate populations, and they exhibit a significant fitness advantage in co-culture with the WT.

A link between RpoS function and QS has been evaluated by multiple studies, with largely discordant findings. Two early studies showed opposing results that *rpoS* is positively regulated by *rhlI* in one and that *rpoS* negatively regulates *rhlI* in the other (40, 41). In another study, microarray analysis showed a 1.8 fold increase in *rhlR* transcript levels during stationary phase growth compared to the *rpoS* mutant, but no evidence of differential regulation of *rhlI* was found (33). Furthermore, if RpoS did repress *rhlI*, we would expect earlier activation of the major casein protease LasB in the *rpoS* mutant, which is inconsistent with the phenotype we observed (Figure 4). Intriguingly *rpoS* mutants do not grow in single culture on casein but outgrow the WT in co-culture (Figure 3,4), indicating a burden that cooperators have to bear. Extracellular proteases do not seem to be responsible for the observed obligate phenotype as there

were no differences in casein proteolysis between the WT and the *rpoS* mutant (Figure 5). This observation is consistent with recent transcriptome data (33). Even though more than 100 genes show co-regulation by QS and *rpoS*, the gene encoding LasB is not among this group. However, another public good, the secreted aminopeptidase PaAP encoded by *pepB* is highly induced by both RpoS and QS (9, 33). We propose that it contributes to the observed strain interaction. PaAP produced by the WT would not contribute to caseinolysis directly but would cleave individual amino acids from peptides that are important for growth, thereby promoting growth of the *rpoS* mutant that itself does not express *pepB*. We also identified a non-social, direct fitness benefit in the *rpoS* mutant. The mutant showed improved growth on a dipeptide (Figure 6), a phenotype that was also observed for the *psdR* mutant. Consistent with this observation, RpoS represses transcription of an amino acid/dipeptide uptake and processing gene cluster (including *mdpA* and the *dpp* genes) during stationary phase (33). This presumably occurs due to sigma factor competition (37). There are a finite number of RNA polymerase complexes in a cell. When RpoS starts coordinating transcription in the cell, this reduces the number of core RNA polymerase interactions that can take place with RpoD, the main housekeeping sigma factor. Transcription of *mdpA* and *dpp* is dependent on RpoD, so increased competition from RpoS would indirectly cause a repression of these loci during stationary phase. In contrast, *rpoS* mutants would express these genes at higher levels in stationary phase. This would lead to an increase in fitness due to better transport and processing of the casein breakdown products, similar to *psdR* mutants. This updated view on the separation of cooperative and non-

cooperative adaptations, resulting in delayed population collapse can be mapped with a simple addition to the earlier diagram (Figures 1,7). Taken together, we propose that *rpoS* affects both cooperative and non-cooperative processes.

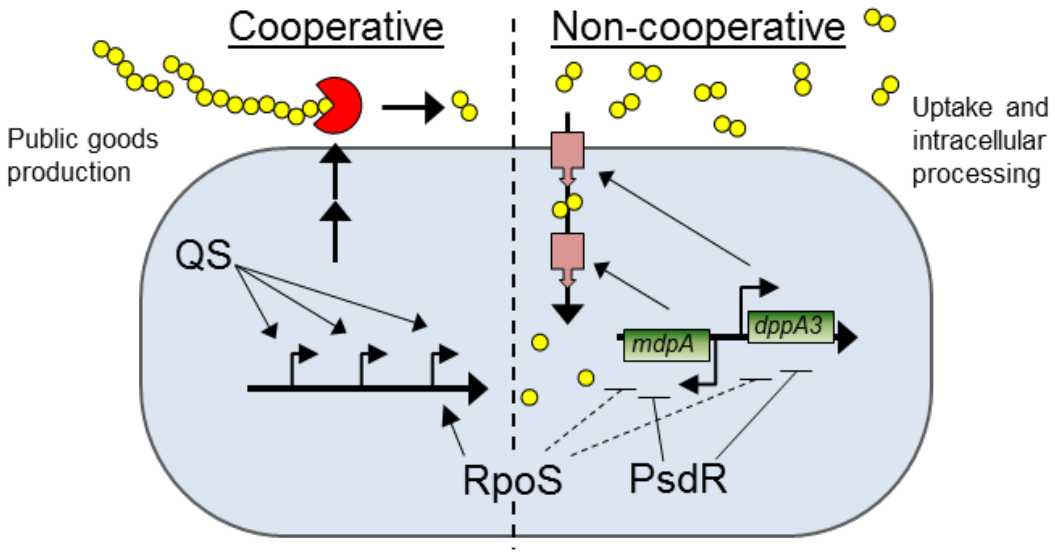

Figure 7 Summary of the cooperative and non-cooperative mechanisms leading to increased growth rate and increased tolerance of cheaters. Extracellular proteases (red) controlled by cooperative QS degrade polypeptides (yellow chains, each circle represents one amino acid) outside the cell. RpoS induces high expression of a QS secreted aminopeptidase, leading to further breakdown of polypeptides. The non-cooperative QS-independent system is involved in the transport and processing of dipeptides. Genes (green) code for proteins (pink) that do the actual transport (DppA3) and intracellular breakdown (MdpA) of dipeptides. A mutated PsdR leads to derepression of the non-cooperative system. RpoS indirectly represses the non-cooperative genes, leading to a further fitness increase.

It is questionable whether mutations in *psdR* and *rpoS* alone would be sufficient to sustain QS-dependent cooperation under our experimental conditions. The *psdR* mutants are themselves susceptible to cheaters that also carry *psdR* mutations, and *rpoS* mutants obligately depend on other cooperator strains. It is plausible that non-conserved mutations primarily found in the hybrid cooperator strains have an additional role in sustaining cooperative growth (Table 2). The skim-milk positive and adenosine-

negative hybrid phenotype itself seems to be caused by partial loss-of-function mutations in *lasR* (15). This might be considered a "streamlining" of the QS regulon to essential functions, thereby minimizing metabolic costs. However, recent quantitative characterization of one such *lasR* mutant revealed that it is in fact also impaired in skim milk proteolysis compared to the WT (15). Thus, the other non-conserved mutations in the hybrid cooperators might result in a non-social fitness benefit that compensates for this impairment.

In summary, we have shown that *rpoS* mutants are selected for among the cooperator populations during *in vitro* evolution on casein. These mutants are unable to grow on their own, but exhibit increased fitness when rare in co-culture. We have shown that RpoS is involved in both the social and non-social response to growth under conditions that favor QS. Overall, these results are consistent with our hypothesis that *rpoS* mutants may help stabilize cooperation, although more experiments are required to confirm this notion.

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