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Letter

Co-regulation of cooperative and private traits by PsdR in *Pseudomonas aeruginosa*

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Abstract

Social interactions profoundly shape the dynamics and functionality of microbial populations. However, mechanisms governing the regulation of cooperative or individual traits have remained elusive. Here, we investigated the regulatory mechanisms of social behaviors by characterizing the fitness of transcriptional regulator PsdR mutants in cooperating *Pseudomonas aeruginosa* populations. In a canonical model described previously, PsdR was shown to solely have a nonsocial role in adaptation of these populations by controlling the intracellular uptake and processing of dipeptides. In addition to these known private traits, we found that PsdR mutants also enhanced cooperation by increasing the production of quorum sensing (QS)-regulated public goods. Although private dipeptide utilization promotes individual absolute fitness, it only partially accounts for the growth advantage of PsdR mutants. The absence of the QS master regulator LasR delayed the appearance of PsdR variants in an evolution experiment. We also demonstrated that the growth fitness of PsdR mutants is determined by a combination of the QS-mediated cooperative trait and the dipeptide metabolism-related private trait. This dual trait is co-regulated by PsdR, leading to the rapid spread of PsdR variants throughout the population. In conclusion, we identified a new social model of co-regulating cooperative and private traits in PsdR variants, uncovering the social and nonsocial roles of this transcriptional regulator in cooperating bacterial populations. Our findings advance the fundamental understanding of bacterial social interactions and provide insights into population evolution, pathogen infection control and synthetic biotechnology.

Keywords: *Pseudomonas aeruginosa*, PsdR, social interaction

Lay Summary

Pseudomonas aeruginosa is a leading cause of opportunistic acute and chronic infections in humans. The establishment and progression of *P. aeruginosa* infections are significantly influenced by the intricate social dynamics of its subpopulations. We uncover a new mechanism of social interaction in which cooperative and private traits are co-regulated by PsdR, a transcriptional repressor of the XRE-cupin family. Our investigation demonstrates that the inactivation of PsdR heightens quorum sensing (QS) activity with increased production of shared public goods and facilitates intracellular dipeptide utilization. Given that PsdR is a regulator governing both cooperative and private traits, our findings suggest that PsdR may serve as a potential target for manipulating social interactions within the *P. aeruginosa* population. This discovery provides a new perspective for the control of pathogen infection and opens doors to synthetic applications.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen known for its ability to cause severe infections, especially in people with compromised immune systems (Gellatly & Hancock, 2013; Klockgether & Tümmler, 2017). This bacterium often exists in complex communities, in which individual cells communicate, compete, and cooperate (West et al., 2007). Social interactions among *P. aeruginosa* cells can significantly influence dynamics of infections and community structures (Jousset et al., 2013; Köhler et al., 2009). One type of cooperation involves cells secreting extracellular "public goods," which are shared among and confer benefits to the entire population. Many important functions, such as biofilm formation, nutrient acquisition, iron chelation, and cell–cell communication, rely on public goods (Nadell et al., 2008; Velicer, 2003; West et al., 2007). On the other hand, this type of cooperation is susceptible to cheating by cells that avail themselves of public goods without contributing to their production. Cheaters gain a growth advantage compared to cooperators by avoiding the metabolic costs associated with public goods production (Nowak, 2006; West et al., 2002). As the number of cheaters escalates, public

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goods are depleted, which can ultimately lead to the collapse of the entire population (Keller & Surette, 2006; West et al., 2006). However, co-existence of cooperators and cheater cells is a common phenomenon (Bruger & Waters, 2015), suggesting that there are underlying mechanisms that are responsible for sustaining cooperation within the bacterial community. We are interested in factors that interact with and stabilize cooperation within populations. In this study, we focus on the interactions between quorum sensing and mutants of a transcription factor called PsdR, which represses dipeptide transport. Others have suggested that the emergence of *psdR* mutants in cooperating populations is due to selection for private functions (like dipeptide catabolism), but our data reported below demonstrate that such private functions cannot explain overall selective advantages of these mutants and highlight additional quorum sensing functions that are impacted in these mutants.

Studies examining cooperators and cheaters have unveiled diverse mechanisms that bolster cooperation while safeguarding against exploitation by cheaters. These mechanisms ultimately enhance the relative fitness of cooperators. For example, partial privatization of public goods prioritizes access to producing cells (Craig Maclean & Brandon, 2008; Gore et al., 2009; Koschwanez et al., 2013). Production of public goods regulated by quorum sensing (QS) enables cooperative cells to produce these goods only when the cell density reaches a threshold. QS regulation of such goods can reduce the exploitation by cheaters within the population by diminishing their relative fitness advantage (Asfahl & Schuster, 2017; Darch et al., 2012). A phenomenon called metabolic prudence allows the production of public goods on conditions where cell division is unlikely, reducing the metabolic cost borne by cooperators (Xavier et al., 2011). Kin discrimination further promotes cooperation by favoring highly related individuals, while spatial structuring restricts the diffusion of public goods and increases the benefits for cooperators (Kümmerli et al., 2009; Lion & Baalen, 2008). Conversely, cooperation can be stabilized by diminishing the fitness of cheaters. Policing mechanisms, such as the secretion of toxins, effectively curb cheater expansion, while cooperators remain immune and resistant (Castañeda-Tamez et al., 2018; Manhes & Velicer, 2011; Wang et al., 2015). Private goods have been observed to confine cheaters under specific nutritional circumstances. These types of mechanisms collectively restrict the spread of cheaters, thereby maintaining cooperation within populations.

P. aeruginosa employs a complex cell–cell communication system called quorum sensing, which involves multiple signals and receptors. There are two well-characterized acyl-homoserine lactone (AHL) systems in *P. aeruginosa*: the *las* and *rhl* systems. These systems are hierarchically organized, with the *las* system regulating the *rhl* circuit (Schuster & Greenberg, 2006). In the *las* system, the signal synthase LasI generates the *las* signal molecule, which is recognized by the receptor LasR, a transcription factor. LasR activates dozens of genes, including the one encoding RhlR, the receptor for the *rhl* signal molecule signal synthesized by the paired synthase RhlI. Additionally, there is also a third QS circuit, the PQS system, which involves a quinolone signal (Diggle et al., 2006; Pesci et al., 1999). QS is known to be a mechanism for bacterial cooperation (Bruger & Waters, 2016). It controls the production of various secreted virulence factors (West et al., 2006), such as the extracellular protease elastase (Sandoz et al., 2007) and (in other bacteria) iron-scavenging siderophores (Griffin et al., 2004), in a cell density-dependent manner (Papenfort & Bassler, 2016). These extracellular products function as public goods, benefiting all the members of the population (Diggle et al., 2007; Sandoz et al., 2007; West et al., 2006). QS also induces the production of some intracellular private goods, such as nucleoside hydrolase (Nuh), which enables bacteria growth on adenosine (Dandekar et al., 2012). The co-regulation of public goods and private goods by QS reduces the selective pressure favoring mutants deficient in private goods under specific growth conditions (Dandekar et al., 2012).

In addition to the QS circuits, the *P. aeruginosa* genome encodes a diverse array of XRE-cupin transcriptional regulators, all characterized by the presence of a helix-turn-helix xenobiotic response element (XRE) domain coupled with a cupin sensor domain. XRE-cupin proteins typically suppress the expression of nearby genes that are involved in condition-specific metabolic pathways (Trouillon et al., 2021). One such member of this XRE-cupin family is PsdR (Kiely et al., 2008; Trouillon et al., 2021). Similar to other XRE-cupin regulators, PsdR has been described to operate as a local transcriptional regulator. PsdR directly binds to the promoter regions of two proximate genes(Trouillon et al., 2021), namely *mdpA*, which encodes a metallo-dipeptidase (Kiely et al., 2008) and *dppA3*, responsible for the transport of small peptides (Pletzer et al., 2014). Through its regulation of *mdpA* and *dppA3* (Asfahl et al., 2015; Kiely et al., 2008; Trouillon et al., 2021), PsdR regulates the intracellular transport and processing of dipeptides.

Mutations in *psdR* that lead to an inactive protein commonly appear when *P. aeruginosa* PAO1 is cultivated in casein broth. In this environment, QS activation is required for the production of proteases that are essential for cell growth (Asfahl et al., 2015; Smalley et al., 2022). Remarkably, PsdR variants arise quickly and become dominant within the population, even before the emergence of cheating LasR mutants. Asfahl et al. described these PsdR mutants as conferring a "nonsocial" fitness advantage in casein broth (Asfahl et al., 2015). PsdR variants were favored within a population due to heightened absolute fitness by improving the utilization of intracellular dipeptides. This trait equips PsdR variant populations with an increased tolerance for cheater populations and delays the occurrence of the "tragedy of the commons" (Asfahl et al., 2015). These effects of PsdR variants in the evolving population were not found to be associated with the QS circuit (Asfahl et al., 2015). However, we speculated that such a nonsocial role of PsdR variants may not provide a comprehensive explanation for their emergence and expansion in an environment reliant on QS activation.

PsdR has recently been demonstrated as a QS negative regulator (Qiu et al., 2024). Inactivation of PsdR activates the QS circuit through derepressing the QS master regulator gene *lasR*. This finding led us to predict that PsdR may in fact influence QS-related cooperative behavior, in addition to the previously reported nonsocial benefit. In the present study, we identified a new mechanism of social regulation responsible for the rapid fixation of PsdR variants within an evolving population. We found that in addition to the known involvement in intracellular dipeptide metabolism, PsdR mutants also promoted the production of QS-controlled public goods. While previous studies have suggested that the emergence of PsdR mutants is driven by selection for private functions, such as dipeptide catabolism, our data demonstrate that these private functions alone do not account for the overall selective advantage of these mutants in casein media. Our study showed that both QS-mediated cooperation and dipeptide-related private behavior collectively contribute to the growth advantage observed in PsdR mutants. More intriguingly, the cooperative and private traits are co-regulated by PsdR. Our

findings suggest that PsdR has both social and nonsocial roles in cooperating populations of *P. aeruginosa*.

Results

PsdR-null mutations enhance cooperative behavior independent of private dipeptide utilization

We hypothesized that PsdR may regulate population cooperation due to its association with the QS circuit. To investigate this hypothesis, we conducted an examination of QS activity and QS-controlled public goods in PsdR-null strains, as compared to the wild-type strain PAO1. We first ensured that all strains showed similar growth rates (Supplementary Figure S1). Consistent with recent findings (Qiu et al., 2024), PsdR-null mutants exhibited substantially elevated QS activity, as indicated by the heightened fluorescence level of three distinct QS reporters (P*lasR*-GFP, P*rhlA*-GFP, and P*pqsA*-GFP) (Figure 1), which measures *las*, *rhl*, and PQS activity, respectively. Surprisingly, deletion of *mdpA* and *dppA3* in PsdR-null mutants had no discernible impacts on their increased QS activities (Figure 1). This observation suggested that PsdR-mediated QS regulation is independent of its influence on dipeptide utilization. We next quantified QS-dependent public goods in PsdR-null derivatives. We found a notable increase in the production of QS-regulated extracellular products, including the protease elastase and phenazine pyocyanin, in the PsdR-null strains as compared with the wild-type strain (Figure 2). As expected, overexpression of PsdR in the PsdR-null mutant (Δ*psdR*::P*rrnB*::*psdR*) markedly diminished the QS activities of *las*, *rhl* and PQS systems (Figure 1). This reduction also reflected in the decreased production of QS-dependent public goods (Figure 2). These findings suggest that PsdR-null mutations not only facilitate private dipeptide utilization but also elevate the production of extracellular public goods through the activation of the QS system.

Improved dipeptide utilization does not fully explain to the competitive advantage of PsdR mutants

We next asked whether the previously established dipeptide pathway, involving *mdpA* and *dppA3*, underpins the enhanced growth fitness of PsdR-null mutants in competition with PsdR-intact cells. To do so, we carried out a competition experiment to determine the relative growth advantage of PsdR derivatives grown in casein broth. Under our test conditions, strains lacking PsdR outcompeted their wild-type counterparts (Figure 3), consistent with previous findings (Asfahl et al., 2015). To probe the role of dipeptide regulation in this growth advantage, we deleted the *mdpA* and *dppA3* genes from both the wild-type strain (resulting in Δ*mdpA*Δ*dppA3*) and the PsdR-null mutant (resulting in Δ*psdR*Δ*mdpA*Δ*dppA3*). While the elimination of the dipeptide metabolism pathway did significantly reduce the growth fitness of Δ*psdR*Δ*mdpA*Δ*dppA3*, it nevertheless retained a significant advantage over Δ*mdpA*Δ*dppA3* (Figure 3). These results suggested that the utilization of private dipeptides only partially accounts for the competitive advantage observed in PsdR-null mutants; PsdR-null mutants appear to employ a hitherto unidentified mechanism to heighten their growth advantage.

QS-mediated growth advantage in PsdR-null mutants

QS plays a pivotal role in microbial social interactions (Allen et al., 2016; Bruger & Waters, 2016). For instance, QS-controlled production of pyocyanin and hydrogen cyanide constrains the expansion of cheaters (Castañeda-Tamez et al., 2018; Wang et al., 2015). Based on our observation of QS activation induced by inactivation of PsdR, we reasoned that QS activation may confer a competitive advantage to PsdR-null mutants. To investigate this possibility, we deleted the master QS regulator *lasR* gene and examined its impacts on the growth advantage of PsdR-null mutants. Given that LasR-null mutants grow poorly in casein broth, we performed competition experiments in a minimal medium containing casamino acids (CAA). As CAA is the product of hydrolyzed casein; dipeptide utilization is not a limiting factor for bacterial growth in this medium. In this environment, PsdR-null mutants continued to outcompete PsdR-intact strains (Figure 4), supporting the notion that the role of PsdR in competition does not fully depend upon private dipeptide utilization. By contrast, when the LasR QS system was removed, the growth fitness of PsdR-null mutants markedly decreased (Figure 4). Deletion of *mdpA* and *dppA3* yielded similar competitive results (Figure 4), demonstrating that QS-mediated growth fitness is independent of the dipeptide metabolism pathway. Taken together, these experiments showed that both the QS circuit and private dipeptide utilization account for the growth advantage of PsdR-null mutants.

Emergence of PsdR mutants correlates with QS activation in casein broth

As discussed above, mutations in *psdR* commonly arise when *P. aeruginosa* is grown in casein broth (Asfahl et al., 2015; Smalley

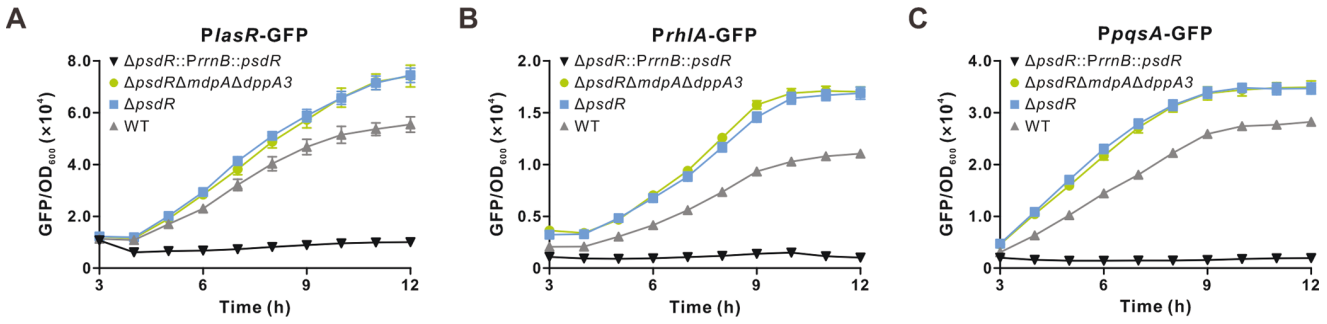

Figure 1. The expression of PsdR influences QS activity independently of dipeptide metabolism. P*lasR*-GFP (A), P*rhlA*-GFP (B) and P*pqsA*-GFP (C) reporter plasmids were mobilized into target strains. Strains-bearing reporters were grown in CAA medium at 37 °C. The expression level of GFP in each strain was quantified using a microreader and reported as relative fluorescence units divided by OD_{600}. Data are presented as mean ± SD ($n \geq 7$). In some circumstances, the error bars are too small to be seen. WT, wild-type PAO1; Δ*psdR*, PsdR-null mutant; Δ*psdR* Δ*mdpA* Δ*dppA3*, triple gene deletion mutant; Δ*psdR*::P*rrnB*::*psdR*, PsdR-null mutant carrying a single copy of *psdR* driven by the *rrnB* promoter.

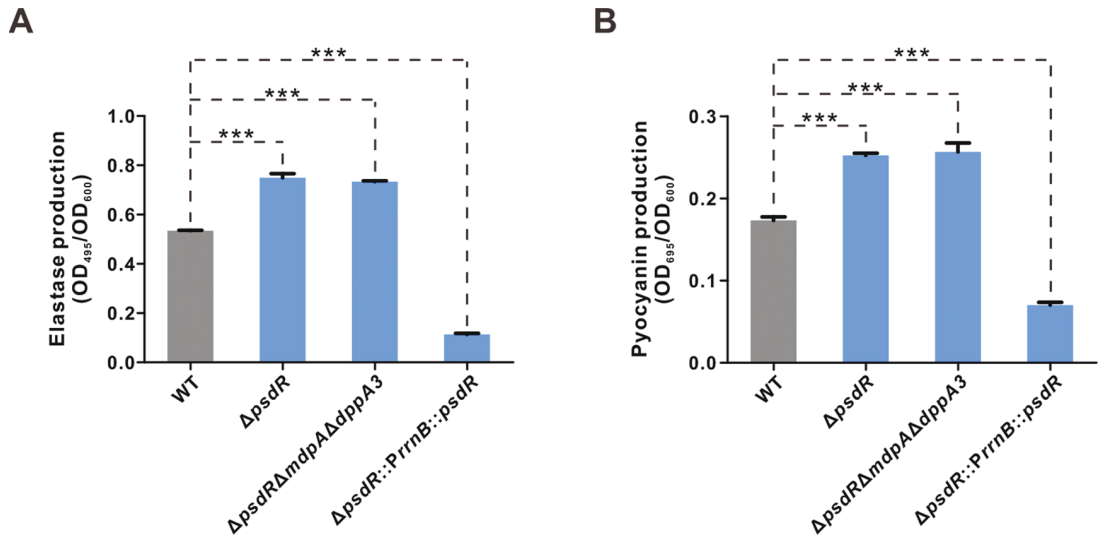

Figure 2. PsdR expression influences the production of QS-regulated public goods. Quantification of QS-regulated public goods in the designated strains. Elastase production (A) and pyocyanin production (B) were reported as OD_{495} or OD_{695} divided by cell density (OD_{600}). Data are presented as mean ± SD ($n = 4$). ***$p < 0.001$ by one-way ANOVA with Bonferroni's correction applied.

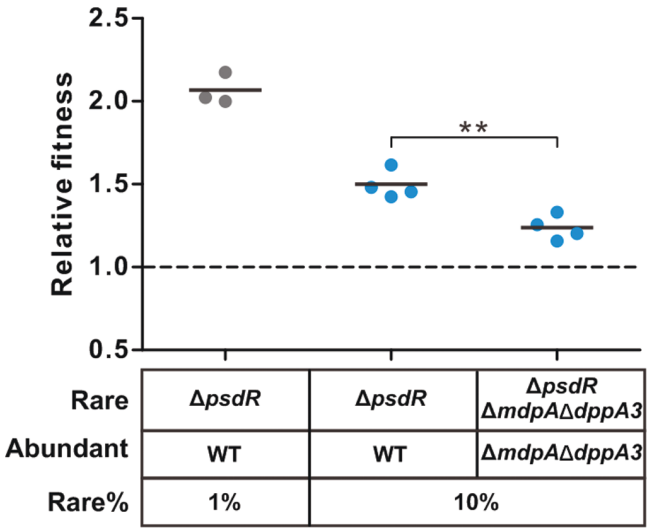

Figure 3. Intracellular dipeptide utilization only partially accounts for the relative growth fitness of PsdR-null. Each PsdR-null derivative labeling with Gm-resistance (pUC18T-mini-Tn7T-Gm) was co-cultured with the designated strain at a start ratio of 1:99 or 10:90 and grown in casein broth for 24 hr. Colonies were enumerated, and relative fitness was computed as the ratio of Malthusian growth parameters (w). **$p < 0.01$ by t-test.

et al., 2022). Based on our finding that PsdR functions as a negative QS regulator by directly targeting *lasR*, we reasoned that *psdR* mutations may closely correlate with QS activation in this environment. We hypothesized that a deletion of the *lasR* gene may delay the emergence of *psdR* mutations in casein. To test this hypothesis, we conducted an evolution experiment and monitored the emergence of *psdR* mutations in strains with or without *lasR*. Consistent with the Asfahl study (Asfahl et al., 2015), wild-type PAO1 acquired *psdR* mutations in all evolved lines by day 6 (Supplementary Table S1). Sequencing of these isolates revealed either single nucleotide substitutions or deletions in *psdR*, resulting in amino acid changes. In contrast, no *psdR* mutations were detected in a LasR-null background mutant even by day 6. As LasR-null cells are deficient in protease production and thus grow poorly in monoculture in casein broth, the lack of *psdR* mutations in this strain might be attributed to the relative lack of growth. To address this concern, we grew an elastase (LasB)-null mutant in casein broth. The LasB-null strain retains LasR QS but is defective in protease production, leading to a comparable growth between the LasB-null and LasR-null strains. Neither strain grows well in casein, as indicated by the enumeration of colony formation units (Supplementary Figure S2). After sequencing *psdR* in isolates collected at day 6, we found that the LasB-null mutant acquired *psdR* mutations far more frequently than a LasR-null mutant (Supplementary Table S1 and Supplementary Figure S3), although the *psdR* mutations we observed in the LasB-null background were different than in the wild-type. These results indicate a close correlation between the emergence of *psdR* mutations and the presence of an intact Las QS circuit in casein broth. Furthermore, since *psdR* mutations appeared before other mutations in the evolved population (Asfahl et al., 2015), our results support the view that PsdR inactivation is important for subsequent QS activation in this environment.

Co-regulation of public and private traits by PsdR

PsdR functions as a transcriptional repressor, directly modulating the expression of *lasR*, as well as two dipeptide-related genes *mdpA* and *dppA3* (Qiu et al., 2024). We inferred that PsdR may co-regulate both the QS-mediated public cooperative trait and private dipeptide utilization trait. To explore this hypothesis, we conducted a comparative analysis of the expression levels of relevant genes in response to variations in PsdR expression. We used a GFP reporter system (designated as P*lasR*-GFP and P*dppA3*-GFP) to measure the transcription of *lasR* and *dppA3*, respectively. As expected, we observed significantly higher levels of fluorescence in PsdR-null mutants compared with wild-type strains, indicating an upregulation of both *lasR* and *dppA3* genes in response to the elimination of PsdR expression (Figure 5). These gene expression patterns demonstrate that PsdR co-regulates the QS-mediated cooperative trait and dipeptide-related private trait. Private goods have been found to be regulated by QS (Dandekar et al., 2012; Heurlier et al., 2005; Schuster et al., 2003). Our work indicates that both a QS-mediated cooperative trait and private trait in a cooperating population can be co-regulated by PsdR.

Illustration of the role of PsdR in QS evolution

We propose an integrated model that combines the private trait previously identified and the QS-mediated cooperative trait newly discovered in the present study (Figure 6). In our model, PsdR variants effectively co-regulate both the QS circuit and the dipeptide pathway. In brief, in environments favoring QS activation such as in casein broth, inactive PsdR derepresses the transcription of the QS regulator gene *lasR*. This leads to the activation of the QS system and a subsequent increase in QS-mediated cooperative behaviors. Simultaneously, the decreased levels of PsdR derepress the expression of *mdpA* and *dppA3*, facilitating the intracellular uptake and processing of dipeptides. However, when bacteria encounter conditions where QS activation is dispensable, the heightened expression of PsdR effectively silences both the QS circuit and the dipeptide pathway. This remarkable dual trait affords PsdR variants a significant growth advantage, enabling them to emerge early and rapidly dominate throughout the entire population during QS evolution in casein broth.

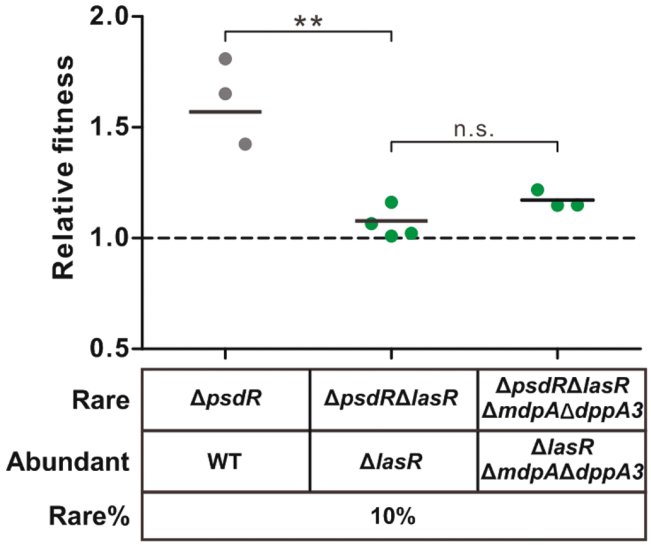

Figure 4. QS contributes to the relative growth fitness of PsdR-null. Each Gm-labeling PsdR-null derivative was co-cultured with the shown strain at a start ratio of 10:90 and grown in CAA medium for 24 hr. Relative fitness was determined as the ratio of Malthusian growth parameters (w). $^{**}p < 0.01$ by one-way ANOVA with Bonferroni's correction applied. *n.s.*, not significant.

Discussion

We describe a new social role for PsdR in the evolution of *P. aeruginosa*. Our findings show that QS confers a relative growth advantage to PsdR mutants over the wild-type strain. Our results are consistent with the established understanding of the role of QS in controlling the production of public goods and cooperative traits within microbial communities (Allen et al., 2016; Bruger & Waters, 2016). In a prior study, a nonsocial role of PsdR variants in QS evolution was described, resulting in the delay of a "tragedy of the commons" (Asfahl et al., 2015). Specifically, PsdR was shown to be unrelated to the QS regulation pathway and influence individual fitness solely by controlling the intracellular utilization of dipeptides (Asfahl et al., 2015; Trouillon et al., 2021). However, our research suggests that these data were incomplete and focuses on the private goods utilization by PsdR, without considering a role in public goods regulation (Asfahl et al., 2015), and does not fully explain the rapid emergence and proliferation of PsdR variants in a growth condition that requires QS-dependent public goods. By systematically examining the growth fitness of PsdR mutants, we demonstrated that both the QS circuit and the dipeptide metabolism substantially contribute to the growth advantage of PsdR mutants in the context of QS evolution. Like other genetic adaptations identified in the QS evolution, such as LasR and MexT mutants (Diggle et al., 2007; Kostylev et al., 2019; Oshri et al., 2018; Sandoz et al., 2007), PsdR variants also closely associate with QS regulation. Furthermore, our study revealed that deletion of the QS gene *lasR* deferred the appearance of PsdR variants, implicating a QS adaptive role for PsdR variants. Therefore, we suggest that genetic adaptations arising in QS-dependent growth environments, such as casein broth, most likely center around QS modulation.

In addition to QS-controlled extracellular products (Diggle et al., 2007; Sandoz et al., 2007; West et al., 2006), bacterial QS orchestrates the expression of multiple genes coding for intracellular products. These private goods include various cellular components, such as Nuh known to catalyze adenosine and inosine (Heurlier et al., 2005), catalase and superoxide dismutase essential for relieving oxidative stress (Hassett et al., 1999) and cytochrome *c* oxidase required for immunity to self-produced

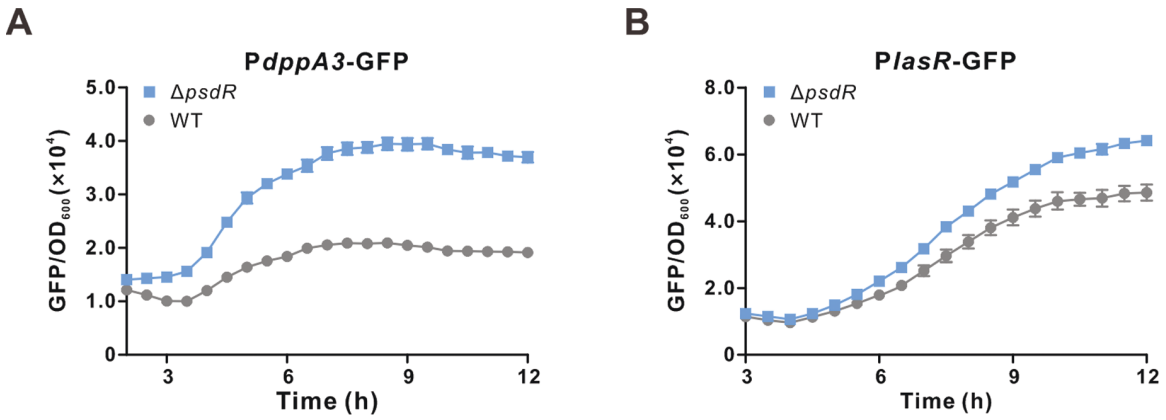

Figure 5. Inactivation of PsdR increases the expression of both *lasR* and *dppA3* genes. The PsdR-null mutant shows increased transcription of *dppA3* (A) and *lasR* (B) genes as estimated by perspective promoter-GFP fusion reporter constructs, P*mdpA3*-GFP and P*lasR*-GFP. Strains-bearing reporters were grown in CAA medium at 37 °C. Data are shown as mean ± SD ($n = 6$). In some cases, the error bars are too small to be seen.

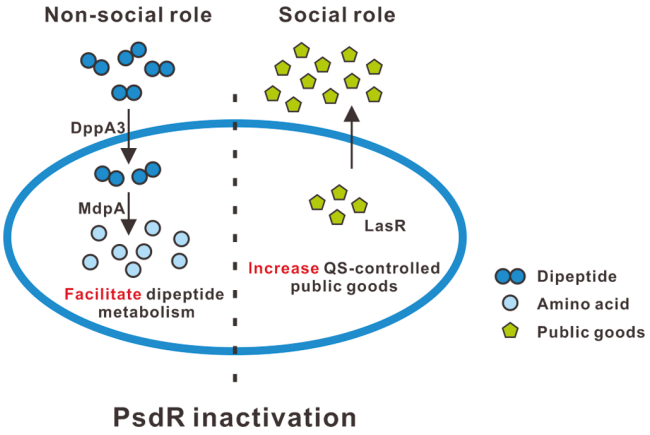

Figure 6. Diagram summarizing co-regulation of cooperative and private traits by PsdR. Diagrams illustrating that inactive PsdR activates QS circuit and QS-controlled public goods production through the derepression of *lasR* transcription and facilitates the dipeptide uptake and processing by increasing the expression of *mdpA* and *dppA3* genes. The co-regulation of cooperation and private behavior suggests PsdR variants possess both social and nonsocial roles in *P. aeruginosa* QS evolution.

hydrogen cyanide (Cunningham et al., 1997). The control of these private goods by QS has led to the proposition that they are directly or indirectly associated with cooperative behaviors in an ecological context (Dandekar et al., 2012; Schuster et al., 2017). More broadly, the link between public and private traits has been suggested to contribute to cooperation (Dandekar et al., 2012; Dos Santos et al., 2018; Foster et al., 2004). However, this relationship typically occurs under very restrictive conditions. Consistent with this idea, our present investigation identified and characterized a social mechanism that links cooperative and private traits under a specific growth condition: casein broth, a protein-based medium where a QS-controlled protease is essential for bacterial growth (Diggle et al., 2007; Sandoz et al., 2007). Unlike the QS-dependent private goods identified previously, we found that a QS-independent private good, dipeptide uptake and processing regulated by MdpA and DppA3 (Kiely et al., 2008; Trouillon et al., 2021), is co-regulated with QS by PsdR. Our work shows that adaptations in *psdR* during QS evolution in casein broth resulted in activation of both QS and dipeptide metabolism. We propose that this co-regulation confers PsdR variants with both increased fitness as cooperators and as individuals. Consequently, PsdR-null mutants gain a competitive advantage over cells containing functional PsdR. Uncovering this new co-regulation mechanism provides an explanation for why *psdR* mutations emerge very early and rapidly dominate wild-type PAO1 populations when passaged in casein broth (Asfahl et al., 2015), but why *psdR* mutations were not detected in LasR-null populations that evolved to grow on casein (Kostylev et al., 2019). This unexpected link also demonstrates that regulation of private goods by Qs- or non-QS transcriptional factors leads to different population outcomes—for instance, the regulation of nucleoside catabolism by QS results in an improvement in relative fitness, but not absolute fitness, for cooperators, while the PsdR-linked private trait enhances population productivity (Asfahl et al., 2015) and therefore overall fitness.

In addition to these laboratory experiments, PsdR variants have been identified in various *P. aeruginosa* isolates obtained from diverse clinical sources (Qiu et al., 2024). This observation suggests that these variants are preferentially selected in specific contexts, underscoring their potential role in the adaptation and persistence of *P. aeruginosa* in certain clinical environments. The wide distribution of PsdR variants in natural environments implicates that the unique combination of public and private regulation may provide them with a growth advantage. We suggest that disrupting or uncoupling this co-regulation mechanism has potential for designing novel antivirulence drugs and aiding in the control of infections. Moreover, given that our findings demonstrate that PsdR controls the on/off switch for controlling cooperative and individual traits, modulating PsdR expression levels may offer an alternative therapeutic approach. In addition to the medical implications, our findings also have implications for synthetic biology. The QS-controlled expression of public goods is often linked to the production of various extracellular enzymes in synthetic biological applications. Therefore, maintaining or enhancing bacterial cooperation with the help of improved individual growth fitness would be expected to enhance the production of these enzymes in biotechnological contexts. Taken together, the identification and characterization of the PsdR-controlled co-regulation mechanism in our current study have broad implications for both managements of pathogenesis and bioengineering applications.

Methods

Bacterial strains and growth

P. aeruginosa was routinely grown in lysogeny broth (LB) or LB broth buffered with 50 mM 3-(N-morpholino) propanesulfonic acid, pH 7.0 (LB-MOPS broth). In some specific experiments, *P. aeruginosa* was grown in M9 medium (Sandoz et al., 2007) supplemented with 1% sodium caseinate (C8654, Sigma-Aldrich, New Zealand) (casein broth) or 0.5% casamino acids (CAA medium) as the sole source of carbon and energy. *Escherichia coli* was grown in LB. All broth cultures were grown at 37°C with shaking at 250 rpm in 14-mm FALCON tubes (for 3 ml cultures) unless otherwise specified. Colonies were grown on LB agar or *Pseudomonas* Isolation agar (PIA) (1.5% agar). The concentrations of antibiotics used in this study were gentamicin 50 μg/ml, ampicillin 100 μg/ml and kanamycin 100 μg/ml. Bacterial strains used in this study are listed in Supplementary Table S2.

Construction of *P. aeruginosa* mutants

P. aeruginosa mutants were constructed using homologous recombination exchange as previously described (Rietsch et al., 2005). For gene knockout construction, 500–1,000 bp DNA flanking of the target gene was generated by PCR and ligated to pGEX2(Gentamycin resistance, Gm) (Rietsch et al., 2005; Stover et al., 2000) (digested with *Hind*III and *Xho*I) with the Vazyme ClonExpress II One Step Cloning kit (Vazyme Biotech, Nanjing, China). The knockout constructs were transformed into the *P. aeruginosa* by triparental mating with the help of PRK2013 strain (Kanamycin resistance, Km). Candidate mutants were first screened on PIA containing 100 μg/ml gentamicin and counter selected on LB agar containing 10% sucrose. Deletion mutants grown on PIA but not PIA Gm were verified by Sanger sequencing. Primers used in this study are listed in Supplementary Table S3.

Reporter assay

QS activity and *dppA3* activity were detected by fusing the promoter of target gene with PROBE plasmid, which contains a gene encoding green fluorescence protein (GFP). QS reporter plasmids (P*lasR*-GFP, P*rhlA*-GFP, P*pqsA*-GFP) and P*dppA3*-GFP were transferred into *P. aeruginosa* PAO1 strains by mating with PRK2013,

and selected on the PIA plate (Gm100). For the gene expression level detection, PAO1 strains bearing target reporter were cultured overnight in LB-MOPS broth (Gm50) and diluted to LB-MOPS broth (Gm50) with OD_{600} of 0.01. Diluted cultures were grown to mid-log phase ($OD_{600} \approx 0.5$) and back diluted to CAA medium with OD_{600} of 0.01 and transferred to 96-well plates (200 μl/well) with six technique replicates. The fluorescence (excitation 488 nm, emission 525 nm) and optical density (OD_{600}) of the samples were recorded every 0.5 or 1 hr for 12 hr by a microplate reader machine (Synergy H1MF, BioTek Instruments, Winooski, VT, USA). The primers used for indicated reporter constructs (P*lasR*-GFP, P*rhlA*-GFP, P*pqsA*-GFP, P*dppA3*-GFP) are listed in Supplementary Table S3.

Elastase production

P. aeruginosa strains were grown in LB broth overnight and diluted with fresh CAA medium ($OD_{600} \approx 0.02$). The 3 ml diluted strains were cultured at 37°C for 24 hr with shaking at 250 rpm. The cells were centrifuged for 2 min at 13,000 rpm, and 500 μl supernatants were transferred to a tube containing an equal amount of ECR buffer (0.1 M Tris-HCl, 1 mM $CaCl_2$, 2.5 mg/ml Elastin-Congo red, pH 7.2) for a light-free mixing reaction at 37°C for 24 hr with shaking. The reaction was stopped by 100 μl 0.12 M EDTA. The insoluble ECR was removed by centrifugation at 5,000 g and 4°C for 5 min, and the OD_{495} of the supernatant was measured. Elastase production was determined by OD_{495}/OD_{600}.

Pyocyanin measurement

P. aeruginosa strains cultured overnight in LB broth were diluted into 4 ml of fresh CAA medium with $OD_{600} \approx 0.02$. The diluted CAA medium was grown at 37°C for 24 hr. Cells were collected at 13,000 rpm for 2 min, and OD_{695} of 200 μl supernatant and OD_{600} of 200 μl corresponding CAA culture were detected by a microplate reader machine with six technique replicates. Pyocyanin production was determined by OD_{695}/OD_{600}.

Evolution experiments

Six independent colonies of the assayed strains were inoculated separately into 3 ml of LB-MOPS broth and incubated overnight. The evolution experiments began by transferring 100 μl overnight cultures into 3 ml of casein broth at 37°C for 36 hr. Subsequently, the evolution was carried out daily by transferring 50 μl of the bacterial suspension into 3 ml of fresh casein broth. Cells were harvested at indicated time points, and single colonies were isolated on LB agar plates. Five clones from each cell line were randomly selected for PCR amplification of the *psdR* coding sequence and subjected to Sanger sequencing.

Competition experiments

P. aeruginosa strains were grown in LB-MOPS broth for overnight and adjusted to an initial $OD_{600} \approx 0.02$ in CAA medium or casein broth. The strains tagged with pUC18T-miniTn7T-Gm were co-cultured with the unlabeled strains at a ratio of 1:99 or 10:90, with a total volume of 3 ml. The mixed cells were cultured at 37°C for 24 hr. The initial and final cell counts were measured by spread-plating 10-fold dilutions on LB agar plates with or without 10 μg/ml Gm at 0 h and 24 h, respectively. The relative fitness is calculated as the ratio of Malthusian growth parameters $(w) = \ln(X_1/X_0)/\ln(Y_1/Y_0)$ (Lenski et al., 1991).

Statistical analysis

Statistical analyses were performed using Excel, GraphPad Prism5, and R software.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

All data generated during this study are included in this published article and its supplementary information files.

Author contributions

H.Q. carried out the experiments and made the figures, H.Q., A.A.D., and W.D. contributed to the analysis of the model. H.Q. wrote the first draft of the manuscript, and all authors contributed to the final vision of the manuscript. The project was initiated by A.A.D. and W.D.

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Conflict of interest: The authors declare no conflict of interest.

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