Conditional privatization of a public siderophore enables *Pseudomonas aeruginosa* to resist cheater invasion

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Understanding the mechanisms that promote cooperative behaviors of bacteria in their hosts is of great significance to clinical therapies. Environmental stress is generally believed to increase competition and reduce cooperation in bacteria. Here, we show that bacterial cooperation can in fact be maintained because of environmental stress. We show that *Pseudomonas aeruginosa* regulates the secretion of iron-scavenging siderophores in the presence of different environmental stresses, reserving this public good for private use in protection against reactive oxygen species when under stress. We term this strategy “conditional privatization”. Using a combination of experimental evolution and theoretical modeling, we demonstrate that in the presence of environmental stress the conditional privatization strategy is resistant to invasion by non-producing cheaters. These findings show how the regulation of public goods secretion under stress affects the evolutionary stability of cooperation in a pathogenic population, which may assist in the rational development of novel therapies.

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Natural selection and game theory\(^1\)–\(^3\) predict that cooperative behaviors are vulnerable to the exploitation of common resources by selfish individuals because selfish individuals (cheaters) consume common resources to gain benefits without contributing fairly. In this context, selfish individuals are fitter, eventually resulting in the collapse of cooperation\(^4\) when the magnitude of selfish exploitation exceeds the capacity of a cooperative, a situation known as the ‘tragedy of the commons’.\(^5\) By contrast, cooperation mediated by producing public goods is consistently observed in diverse life forms ranging from microbes to social animals; for example, the yeast Saccharomyces cerevisiae secretes enzymes to externally digest sucrose and thus facilitates sucrose metabolism at the group level\(^6\), and the ant Pristomyrmex punctatus collectively contribute their efforts to build and maintain the nest for their housing.\(^7\) Because of their experimental accessibility and the extensive and diverse genomic data available, microbes are used as model organisms by researchers to probe social interactions at the molecular level and examine fundamental aspects of the origins of cooperation in well-defined systems.\(^8\) In past decades, numerous theoretical and experimental investigations have elucidated several mechanisms and processes that promote cooperation in microbes, including kin selection/discrimination,\(^8\) cooperation when surrounded by cooperators/quorum sensing,\(^9\) punishment/policing,\(^10\) pleiotropy,\(^11\) phenotype variation/bistability,\(^11\) spatial self-organization,\(^12\) and adaptation to changing environments.\(^13\)

Microbes have evolved elaborate strategies to adapt to nutrient scarcity and environmental stresses, which is generally believed to increase competition and reduce cooperation among them. For example, molecular mechanisms underlying decision-making enable bacteria to change their phenotypes or tune their gene expression to adjust to environmental or nutritional perturbations,\(^14\) and to counter-attack when they sense nutrient limitation or direct cell damage.\(^15\) We hypothesize that environmental stresses that induce competition may also stimulate the cooperator to compete and thereby to resist cheater invasion, thus in turn promoting the evolutionary stability of those cooperative traits. This hypothesis is supported by the findings of Xavier et al.,\(^20\) who reported that swarming, a form of cooperative motility mediated by rhamnolipid secretion, can be maintained in Pseudomonas aeruginosa because carbon-rich rhamnolipid is secreted only when nitrogen is more limiting than carbon.

To elucidate mechanisms that make cooperators robust to environmental stresses, such as starvation, antimicrobial treatment, or oxidative stresses, we investigated how P. aeruginosa regulates the production and secretion of iron-scavenging siderophores in the presence of different environmental stresses. In addition, we evaluated whether these environmental stresses can facilitate the persistence of bacterial cooperation in this cooperative system.\(^22\)\(^,\)\(^23\) Using the combination of a single-cell tracking technique, experimental evolution, and theoretical modeling, we identified a strategy we term “conditional privatization” that can increase survival and resist invasion by non-producing cheaters in the presence of environmental stresses.

Results
Stress induce the pyoverdine accumulation in the periplasm. We used a spinning-disc confocal microscope with high spatiotemporal resolution to directly image and localize pyoverdine (PVDI), a fluorescent siderophore.\(^24\) We observed that illumination with a violet laser light (405 nm) led to the accumulation of PVDI in the periplasms of single P. aeruginosa cells (Fig. 1a–c and Supplementary Movie 1). The PVDI concentration increased approximately eight-fold within 4 min when illuminance exceeded 6.00 mW cm\(^{-2}\). Fluorescent PVDis were excited when illuminated by the 405 nm laser source. To decouple the imaging of PVDI and the light stimulus of cells, we carefully optimized laser power and exposure time to identify an excitation condition that would not lead to the accumulation of PVDI or affect the growth of cells. Figure 1d and Supplementary Figure 1 indicate that relatively weak illumination (<3.00 mW cm\(^{-2}\)) does not affect bacterial growth and the excitation condition (0.10 mW cm\(^{-2}\)) does not result in PVDI accumulation. Therefore, 0.10 mW cm\(^{-2}\) excitation was applied to image PVDI in this study. The accumulation of PVDI in the bacterial periplasm may arise from two possibilities: (i) a specific response to the stimulus of violet/blue light\(^25\) or (ii) a general response to photon stress that typically leads to reactive oxygen species (ROS) generation in live cells.\(^26\) To assess these two possibilities, next, we used a ROS-specific dye (2′,7′-dichlorofluorescin diacetate, H\(_2\)DCFDA) in situ to monitor the intracellular levels of ROS in single cells stimulated with violet light (≥6.00 mW cm\(^{-2}\)). This illumination led to the accumulation of PVDI in 2 min. ROS was generated within 8 min in single P. aeruginosa cells (Fig. 1e and Supplementary Movie 2), as indicated by bluish green to show green fluorescence, after stimulation with violet light (≥9.00 mW cm\(^{-2}\)). This finding suggests that the accumulation of PVDI is a general response to photon stress rather than a specific consequence of the stimulus of violet/blue light. To further confirm this finding, we used a low dose of tobramycin, an aminoglycoside antibiotic, with concentrations near the minimal inhibitory concentration to treat P. aeruginosa to enable ROS generation.\(^28\) A low dose of tobramycin also led to the accumulation of PVDI through ROS generation in cells (Fig. 1f). This result indicates that different environmental stresses, including photon and antimicrobial stresses, trigger the accumulation of PVDI in the periplasms of P. aeruginosa.

Accumulated PVDI enables survival of bacteria under stresses. It has been reported that siderophore-mediated iron acquisition is required for resistance to ROS stress in the fungus Alternaria alternate.\(^29\) This report motivated us to investigate the mechanism underlying the accumulation of PVDI in P. aeruginosa cells in response to ROS generation. Intracellular reactive species of superoxide (O\(_2^-\)) or peroxide (H\(_2\)O\(_2\)) can damage Fe–S clusters and result in the release of free ferrous ions from the damaged clusters.\(^30\) The released ferrous ions can subsequently catalyze H\(_2\)O\(_2\) to form the most harmful hydroxyl radical (OH\(^-\)) through the Fenton reaction.\(^31\) PVDI accumulated in P. aeruginosa cells may chelate the released ferrous and thereby repress OH- formation, consequently protecting cells from oxidative damage. To directly validate this conjecture, we used a combination of propidium iodide (PI)-staining and ROS staining to examine whether a mutant strain (ΔpvdA) deficient in PVDI production can tolerate the photon or antimicrobial stress used to treat the wild-type strain. Under the treatment of violet light (3.00 mW cm\(^{-2}\)), tobramycin (1.0 μg mL\(^{-1}\)) or gentamicin (2.0 μg mL\(^{-1}\)) in iron-limited conditions, a higher percentage of ΔpvdA cells (18.5% (p < 10\(^{-6}\)), 23.1% (p < 10\(^{-6}\)) or 25.3% (p < 10\(^{-5}\)) generated ROS than that of wild-type cells (1.2%, 9.9%, or 0.7%) (Fig. 2b, Supplementary Fig. 2c, Supplementary Movie 3) and a higher percentage of ΔpvdA cells (15.4% (p < 10\(^{-6}\)), 15.4% (p < 10\(^{-6}\)) or 8.2% (p < 10\(^{-6}\)) were damaged than that of wild-type cells (0.7%, 0.8% or 1.5%) (Fig. 2b, Supplementary Fig. 2d, Supplementary Movie 4). To further explore whether the protective activity of PVDI is due to the repression of the Fenton reaction, we examined whether PVDI can protect the wild-type and ΔpvdA strains from antimicrobial stress in iron-rich conditions. Our results indicate that PVDI does not protect cells by preventing ROS accumulation.
The uptake of exogenous PVDI (ePVDI) allows only the line present data arising from single bacteria and from the average of multiple bacteria, respectively. The distributions of PVDI within 4 min and ΔpvdA cells did not produce PVDI despite illuminations (6.00 mW cm⁻²), where the PVDI is indicated by blue colors. Distributions of PVDI fluorescent intensities or ROS-specific dye H2DCFDA fluorescent intensities or ROS-specific dye H2DCFDA fluorescent intensities (bluish green bar) arising single cell in the presence of antimicrobial stress. To test this prediction, we used the chelation of excess iron is essential for the protective activity of cells from damage; therefore, the protective activity of PVDI declined in iron-rich conditions. These findings demonstrate that the accumulation of PVDI in bacterial periplasms enables P. aeruginosa cells to survive in the presence of different environmental stresses.

Furthermore, we expected that only PVDI accumulated in bacterial periplasms—not secreted PVDI—can protect cells in the presence of environmental stresses. To test this prediction, we exogenously added PVDI (5.0 μM) in the culture media for ΔpvdA cells or a double-mutant strain ΔpvdAΔfpvA deficient in the uptake of exogenous PVDI (ePVDI)³⁵, allowing only the ΔpvdA cells to absorb exogenous ePVDI. We observed that after the absorption of ePVDI, the fraction of ΔpvdA cells that died (0.8% (p = 0.2557) or 1.5% (p = 0.9725)) was similar to that of the wild-type strain in the presence of photon (Fig. 2b and Supplementary Movie 6) or antimicrobial stress (Supplementary Fig. 2b). These findings suggest that P. aeruginosa cells prefer reserving PVDI molecules intra-cellularly rather than secreting these molecules in the presence of antimicrobial stress. To quantify this phenomenon, we used the

**Fig. 1** Environmental stresses trigger the accumulation of a bacterial siderophores in the periplasms of P. aeruginosa. Representative bright-field (gray) + confocal images show that illuminations of violet-laser (405 nm, 6.00 mW cm⁻²) trigger the accumulation of a bacterial siderophores (pyoverdine, PVDI) in wild-type P. aeruginosa within 4 min and ΔpvdA cells did not produce PVDI despite illuminations (6.00 mW cm⁻²), where the PVDI is indicated by blue colors. a) Distributions of PVDI fluorescent intensities along the vermilion line across the bacterium shown in a for the time t = 0, 60, 120, 240 s, from bottom to top. b) Photon-stress dependence of PVDI fluorescence intensity in single cells, where cells were exposed to violet-laser for 2 min. The dots and the line present data arising from single bacteria and from the average of multiple bacteria, respectively. c) Normalized histograms of PVDI (blue bar) fluorescent intensities or ROS-specific dye H2DCFDA fluorescent intensities (bluish green bar) arising single cell in the presence of different photon-stresses (0.75, 3.00, 4.50, 6.00, and 9.00 mW cm⁻²) or in addition of different amount of tobramycin (0.0, 1.0, 2.0, 3.0, and 4.0 μg mL⁻¹), where cells were exposed to violet-laser for 2 or 8 min or tobramycin for 1 or 7 h, respectively. Scale bar for all images are 2 μm.

**Attenuation the efflux of PVDI for private use.** We observed that the concentration of PVDI in the supernatant of bacterial cultures (wild type) negatively correlated with the concentration of tobramycin (Fig. 3a) when we used low dose tobramycin (≤ 1.0 μg mL⁻¹) to treat bacteria. Note that the tobramycin concentration applied in this experiment did not affect the growth of cells (Supplementary Fig. 1). By contrast, the concentration of PVDI accumulated in cells positively correlated with the concentration of tobramycin (Fig. 1e). These findings suggest that P. aeruginosa cells prefer reserving PVDI molecules intracellularly rather than secreting these molecules in the presence of antimicrobial stress. To quantify this phenomenon, we used the...
following rate equation to describe the kinetics of the intracellular concentration of PVDI \( [\text{PVDI}]^i \):

\[
\frac{d[\text{PVDI}]^i}{dt} = P + a[\text{PVDI}]^o - \gamma[\text{PVDI}]^i - \mu[\text{PVDI}]^i,
\]

(1)

where \( [\text{PVDI}]^o \) is the extracellular concentration of PVDI, \( P \) is the productivity of PVDI, \( a \) and \( \gamma \) are the uptake and efflux rates of extracellular and intracellular PVDI, respectively, and \( \mu \) is the growth rate of a cell. Note that (1) exponential growth dilutes the intracellular concentration of PVDI with the constant of \( \mu \) and (2) the ratio \( \gamma/\mu \) of the terms \( \gamma[\text{PVDI}]^i \) and \( \mu[\text{PVDI}]^i \) accurately quantifies how cells allocate their produced PVDI, in which \( \gamma/\mu > 1 \) or \( \gamma/\mu < 1 \) indicates that cells prefer to secrete or reserve PVDI molecules produced by themselves, respectively. To directly

\[ BF \]
\[ PVDI \]
\[ H_2DCFDA \]
\[ PI \]

\[ \Delta pvdA \]
\[ \Delta pvdA \rightarrow \text{exo PVDI} \]

\[ \Delta pvdA \Delta fpvA \rightarrow \text{exo PVDI} \]

\[ 0.5 \ 1 \ 3 \ 19.8\% \ 0.8\% \ 15.4\% \ 0.7\% \ 1.2\% \ 18.5\% \ 1.4\% \ 17.4\% \ 1.8\% \ 19.8\% \]

\[ 0.5 \ 1 \ 3 \]

\[ \Delta pvdA \Delta fpvA \rightarrow \text{exo PVDI} \]

\[ \Delta pvdA + \text{exo PVDI} \]

\[ \Delta pvdA + \text{exo PVDI} \]

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\[ \Delta pvdA + \text{exo PVDI} \]

Fig. 2 Accumulation of pyoverdine in bacteria allows them to survive in the presence of environmental stresses. a Representative bright-field (gray) + multi-color confocal images show that the accumulation or the exogenous addition of PVDI (5.0 μM) enables wide-type P. aeruginosa or \( \Delta pvdA \) mutant to survive in the presence of a stronger photon-stress (3.00 mW cm\(^{-2}\)), where blue, bluish green or vermillion colors represents PVDI, \( H_2DCFDA \) or propidium iodide (PI) fluorescent intensity respectively. \( \Delta pvdA \) or \( \Delta pvdA \Delta fpvA \) represents a mutant that deficient in production of PVDI or deficient in both production of PVDI and uptake of exogenous PVDI, respectively. +exo PVDI represents the exogenous addition of 5.0 μM PVDI. b Photon-stress dependence of PVDI (blue), \( H_2DCFDA \) (bluish green) or PI fluorescent intensity (vermillion) in single cells, where the dots present data from single bacteria. \( \Delta pvdA \) and \( \Delta pvdA \Delta fpvA \rightarrow \text{exo PVDI} \) groups show significantly higher percentage than that of the wide type group, both for \( H_2DCFDA \) channel and PI channel (one-way RM ANOVA versus wide type data, \( p < 10^{-6} \) for \( \Delta pvdA \) and \( \Delta pvdA \Delta fpvA \rightarrow \text{exo PVDI} \)). \( \Delta pvdA \rightarrow \text{exo PVDI} \) group shows no significantly difference with wide type group, both for \( H_2DCFDA \) channel \( (p = 0.8054) \) and PI channel \( (p = 0.2557) \). The black dashed line represents an intensity threshold to distinguish whether the fluorescence intensities arising from single cells is significantly greater than the average. Single cells were exposed to light stimulations for 5 hours. Scale bar for all images are 4 μm.

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measure the value of $\gamma/\mu$ in the presence of environmental stresses, we pre-cultured $\Delta pvdA$ cells ($P = 0$) in the presence of ePVDI (50 $\mu$M) to allow them to absorb sufficient PVDI; we then rapidly removed all ePVDI from bacterial cultures by washing of a fresh medium ($\alpha_{PVDI} = 0$) and recorded the decline of $[\text{PVDI}]$ as well as the growth of cells in situ in the presence of different photon stresses ranging from 0.10 mW cm$^{-2}$ to 3.00 mW cm$^{-2}$. These specific boundary conditions considerably simplify the Eq. (1). In all tested conditions, the decline of $[\text{PVDI}]$ scaled linearly to the time elapsed ($t$) using a semi-logarithmic plot (Fig. 3b), indicating that the decline of $[\text{PVDI}]$ in these $\Delta pvdA$ cells follows first-order kinetics: $[\text{PVDI}]_i(t)/[\text{PVDI}]_i(t = 0) = \exp\left[-(\gamma + \mu)t\right]$. The decay rate ($\gamma + \mu$) of $[\text{PVDI}]$ correlated nonlinearly (Hill-like) with the magnitude of photon stresses (Fig. 3c), indicating that $\gamma + \mu$ decreased considerably only in the presence of stronger photon stresses ($> 0.50$ mW cm$^{-2}$). Next, we evaluated the value of $\mu$ in the presence of different photon stresses (Fig. 3d), which enabled us to calculate directly the value of $\gamma/\mu$. Note that the illumination conditions applied in this experiment did not affect the growth of cells. Figure 3e shows that the Hill-like transition existed in the profile of $\gamma/\mu$, where the value of $\gamma/\mu$ remained at a constant of 3.70 if photon stress was relatively low (< 0.30 mW cm$^{-2}$); however, its value rapidly decreased to 1.05 if photon stress became higher (>1.00 mW cm$^{-2}$).

It has been reported that *P. aeruginosa* uses an efflux pump (PvdRT-OpnQ) composed of PvdR, PvdT, and OpnQ to secrete PVDI to the environment in a specific manner. To further investigate whether the attenuation of PVDI efflux in the presence of environmental stress is caused by the tuning down of the efflux pump of PvdRT-OpnQ, we examined the response of the $\Delta pvdRT-\text{opmQ}$ mutant deficient in PvdRT-OpnQ expression to photon stress. We found that the initial level of intracellular PVDI of $\Delta pvdRT-\text{opmQ}$ mutant was markedly higher than that of wild-type cells (Supplementary Fig. 4), however, light stimulation (3.00 mW cm$^{-2}$) did not result in
Further accumulation of PVDI in ΔpvdRT–ompQ cells. This finding strongly suggests that P. aeruginosa can specifically tune down the efflux pump of PvdRT-OmpQ in the presence of environmental stress. Hence, we conclude that an existing molecular mechanism enables P. aeruginosa cells to tune down the efflux pump of PvdRT-OmpQ to reserve public PVDI for private use in the presence of environmental stress. We term this mechanism ‘conditional privatization’.

Resistance of cheater invasion via conditional privatization. Griffin et al. reported that the cheating strain of P. aeruginosa (which does not secrete PVDI) eventually outcompetes the cooperative strain (wild type) in various iron-limited conditions, thus resulting in the collapse of cooperation. This collapse occurs because the cheater does not produce the metabolically expensive PVDI, but instead exploits the public goods secreted by cooperators. We predicted that conditional privatization enables the cooperative strain of P. aeruginosa to withstand invasion by cheaters in the presence of environmental stresses. To test this prediction, we mixed an equal ratio of cheater ΔpvdA and cooperative (wild type) strains and cocultured them for 30 days to allow them to evolve in the presence of environmental stresses. We adjusted the magnitude of environmental stresses by adding tobramycin to achieve various final concentrations (0.0 to 2.0 μg mL⁻¹) in the culture medium, with the ferric concentration ranging from 1.0 × 10⁻⁵ to 5.0 × 10⁻² μM. We observed that under all iron-limited conditions, the ΔpvdA strain outcompeted the wild-type strain after 15 days in the absence of a stronger environmental stress ([tobramycin] ≤ 0.125 μg mL⁻¹) (Fig. 4a–c); this finding is consistent with that reported in a previous study. By contrast, the wild-type strain outcompeted the ΔpvdA strain after 7 days in the presence of a stronger environmental stress (1.0 ≤ [tobramycin] ≤ 2.0 μg mL⁻¹). This result indicates that the presence of environmental stresses prevent invasion by the cheater strain. The protective activity of intracellular PVDI plays an essential role in the survival of the cheater strain in the presence of environmental stress; however, exogenously added PVDI can protect the cheater strain ΔpvdA in the presence of environmental stress (Fig. 2, Supplementary Fig. 2). This finding indicates that public PVDI can be exploited by cheaters both to acquire iron in iron-limited conditions and protect themselves in the presence of environmental stress. We have summarized and plotted the results of our evolutionary experiments in Fig. 4d. This figure shows that as the environmental stresses intensify, the final evolutionary outcome changes, with the dominance of the cheater changing to that of the cheater.
To determine the role played by conditional privatization in our evolutionary experiments, we built a theoretical model to link environmental stresses to evolutionary outputs by considering the following factors: (1) production of public goods (PVDI) enables the growth of all populations in iron-limited conditions, but this is metabolically expensive and thus reduces the growth rate of producers (Eqs. 2 and 3 in the Methods); (2) conditional privatization enables producers to reserve those public molecules for private use in the presence of environmental stresses, as indicated by a maintained constant of $\gamma/\mu = \text{cons.} \gg 1$ (Eq. 4 in the Methods); and (3) private use of public goods enables producers to survive in the presence of environmental stresses (Eq. 7 in the Methods). Note that our modeling based on solving the differential equations (Eqs. 8–12), but alternative modeling based on the adaptive dynamics framework also could be applied to determine the evolutionary role played by conditional privatization. To directly compare evolutionary outputs arising from conditional privatization, we assumed that producers may employ distinctive strategies in response to environmental stresses (Fig. 4e), including (1) "loyal cooperation" through which a bacterium always secretes public goods despite environmental stresses, as indicated by a maintained constant of $\gamma/\mu = \text{cons.} \gg 1$ (Eq. 4 in the Methods) and (2) "selfishness" through which a bacterium does not secrete public goods, as indicated by a maintained constant of $\gamma/\mu = \text{cons.} \ll 1$ (Eq. 6 in the Methods). Theoretical calculations indicate that our modeling can reproduce evolutionary kinetics (as indicated by solid lines in Fig. 4a–c) and final evolutionary outcomes (as indicated by the color map in Fig. 4d) that are fully consistent with our experimental observations (as indicated by symbols in Fig. 4a–c or d) in all tested conditions. By contrast, our model predicts that bacterial cooperation would eventually collapse if a bacterium always secreted public goods despite environmental stresses (Fig. 5a). After comparing the evolutionary outcomes dominated by different strategies, including conditional privatization (Fig. 4d) and loyal cooperation or selfishness (Fig. 5a), we concluded that conditional privatization in the presence of environmental stresses is a novel mechanism that can prevent the "tragedy of the commons".

**Competition in wild-type and ΔpvdRT—opmQ strain.** First, we examined the growth rate of ΔpvdRT—opmQ strain in different growth conditions to evaluate the cost of privatization of public PVDI. We found that (1) in the iron-limited condition of FeCl₃ = 1.0 × 10⁻³ μM (Supplementary Fig. 5) or FeCl₃ = 0.1 μM, the growth rate of ΔpvdRT—opmQ mutant ($\mu_3 = 0.0032 \text{min}^{-1}$ at FeCl₃ = 1.0 × 10⁻³μM, Supplementary Fig. 8b or $\mu_3 = 0.0035 \text{min}^{-1}$ at FeCl₃ = 0.1 μM, Supplementary Fig. 9b) was slower than that of the wild-type strain ($\mu_1 = 0.0039 \text{min}^{-1}$ at FeCl₃ = 1.0 × 10⁻³μM, Supplementary Fig. 8a or, $\mu_1 = 0.0099 \text{min}^{-1}$ at FeCl₃ = 0.1 μM, Supplementary Fig. 9a); and (2) the exogenous addition of PVDI (EPVDI = 5.0 μM) markedly increased the growth rate of the ΔpvdRT—opmQ strain ($\mu_3 =$
0.0100 min\(^{-1}\) at \(\text{FeCl}_3 = 0.1 \mu M\) + ePVDI = 5.0 μM, Supplementary Fig. 9c) in iron-limited conditions. These results arising from ΔpvdRT – opmQ strain (constitutive privatization) indicated that (1) production of PVDI is metabolically expensive whether produced PVDI is public or private; (2) the secretion of private PVDI is beneficial to bacterial growth in the iron-limited condition; and (3) PVDI constitutive privatization strain can exploit public PVDI to benefit in iron-limited conditions.

Next, we predict the evolutionary outcome of competition between the wild-type (conditional privatization) and ΔpvdRT – opmQ (constitutive privatization) strains using our theoretical model (see Methods). Our calculation indicated that (1) ΔpvdRT – opmQ coexists with the wild-type strain in the presence of ROS stress (Supplementary Fig. 10a); and (2) the relative yield is negative in the coexistence region (Supplementary Fig. 10b). These results are in contrast with those of the competition of ΔpvdA and wild-type strain, in which the relative yield is positive in the region dominated by ΔpvdA cells (Supplementary Fig. 10c, d). There the results indicated that the ΔpvdRT – opmQ strain is not able to invade the wild-type strain in the absence of ROS stress. To further test this, we predicted the evolutionary outcomes of competition among the wild-type (conditional privatization), ΔpvdRT – opmQ (constitutive privatization) and ΔpvdA (cheater strain) strains. Supplementary Fig. 11a and b show that the wild-type strain is favored in the presence of ROS stress; that is, the wild-type strain cannot be invaded by either ΔpvdRT – opmQ or ΔpvdA strain in the presence of ROS stress.

Roles played by private PVDI. To determine the roles played by private PVDI in our evolutionary experiments, we directly calculated the kinetics of the extracellular PVDI concentration ([PVDI]*\(_i\)(t)), the growth rate (\(\mu_i(t)\) or \(\mu_j(t)\)), death rate (\(p_i(t)\) or \(p_j(t)\)), and intracellular PVDI concentration ([PVDI]\(_i\)\(_j\)(t) or [PVDI]\(_i\)\(_j\)(t)) of the cooperator or cheater strain during competition by using our theoretical model. Our calculations indicated that (1) the cooperator strain outcompetes the cheater strain in the presence of environmental stress (i.e., \(\text{FeCl}_3 = 5.0 \times 10^{-5} \mu M\) and Tobramycin = 2.0 μg mL\(^{-1}\), Supplementary Fig. 12a); (2) the domination of the cooperator strain led to the accumulation of extracellular PVDI, resulting in a gradual increase of [PVDI]*\(_i\) over time (Supplementary Fig. 12b); (3) an increase of [PVDI]*\(_i\) led to an increase in the growth rate of both the cooperator and cheater strains (Supplementary Fig. 12c, note that \(\mu_i(t) > \mu_j(t)\) despite their increase); (4) the intracellular PVDI concentration was high in the cooperator strain (Supplementary Fig. 12c) because ROS stress triggers the privatization of PVDI, which further results in the reduction of the death rate of cooperator strain; and (5) the intracellular PVDI concentration in the cheater strain remains at a low level in the initial stage and then gradually increases with increase in [PVDI]*\(_i\) (note that the cheater strain could also tune down the efflux of PVDI in the presence of environmental stress), which results in an increase in the death rate of the cheater strain in the initial stage, which gradually decreases in the later stage. These results indicated that the wild-type strain outcompetes those non-producers in the presence of ROS stress due to protective activity of intracellular PVDI. To further test this interpretation, we predicted the evolutionary outcomes of competition between the wild-type strain (conditional privatization) and ΔpvdA strain (cheaters) with an additional assumption that intracellular PVDI does not enable cells to survive in the presence of ROS stress. We found that under this assumption the cheater always outcompetes the cooperator strain despite ROS stress (Supplementary Fig. 13).

Discussion

We have shown that when \(P.\ aeruginosa\) is exposed to photon and antibiotic stresses, it suppresses secretion of the siderophore PVDI and instead reserves PVDI intracellularly. The intracellular PVDI protects cells from ROS damage and increases cell survival in stressful conditions. We term this strategy ‘conditional privatization’: the bacteria secrete public goods to cooperate with other cells in the absence of environmental stresses, whereas they reserve those public goods for private use once the environment becomes stressful. This conditional strategy outcompetes the non-producing, cheater strategy under environmentally stressful circumstances (Fig. 5b). The molecular mechanism of conditional privatization follows a hierarchical procedure: (1) environmental stresses generally trigger ROS generation in \(P.\ aeruginosa\) cells; (2) ROS triggers cells to tune down the efflux pump of PvdRT – OpmQ to decrease the secretion of PDVI, leading to the accumulation of PDVI in cells; and (3) the accumulation of PDVI enables cells to survive in the presence of environmental stresses, presumably through the repression of hydroxyl radical formation. However, the mechanism through which the efflux pump of PvdRT – OpmQ is tuned down in response to ROS remains elusive, meriting a more detailed investigation.

The private use of public goods by their producer might be a common mechanism that enables the persistence of cooperative behaviors in microbes. For example, Gore et al.\(^{37}\) reported that the budding yeast \(S.\ cerevisiae\) can retain approximately 1% of public goods for private use, and that such a seemingly insignificant level of privatization allows cooperative cells to invade a population of cheaters\(^{37,38}\). In addition, our findings highlight that environmental stresses affect cooperation in microbes, which may be compared to the effect of resource scarcity on cooperation in human society. For example, Gatiso et al.\(^{39}\) found that people who encountered resource scarcity were less cooperative compared with those who had more abundant resources in an investigation of the extraction of trees from a communally managed forest in Ethiopia. It should be emphasized that conditional strategies, including conditional cooperation or cheating, are quite general. For example, Rustagi et al.\(^{40,41}\) reported that conditional cooperation and costly norm enforcement can stabilize large-scale cooperation for common management. By contrast, our findings highlighted that one conditional strategy can coordinate the secretion of PVDI that plays the dual function; i.e., public PVDI for iron scavenging or private PVDI against ROS stress.

As a crucial opportunistic human pathogen, understanding the cooperation of \(P.\ aeruginosa\) is of great significance to clinical therapies\(^{42}\). Exploitation of iron in the hosts of \(P.\ aeruginosa\) depends on the cooperative production of siderophores\(^{43}\) and virulence correlates with siderophore production\(^{44}\). Therefore, our findings of how \(P.\ aeruginosa\) alters siderophore secretion in the presence of environmental stresses can not only advance our understanding of the maintenance of cooperation but also provide an opportunity to deepen our understanding of the relationship between cooperation and virulence. This advance may assist in the rational development of novel therapies for pathogenic populations\(^{45}\).

Methods

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Strains were grown on LB agar plates at 37 °C for 12 h. Monoclonal colonies were inoculated and cultured with a minimal medium (SSM) at 37 °C under an aerobic condition, in which the medium had the following composition per liter: K2HPO4, 7.86 g; KH2PO4, 3.00 g; (NH4)2SO4, 1.00 g; MgSO4·7H2O, 0.20 g; succinic acid, 4.00 g NaOH was added to the medium to adjust pH = 7.0 before sterilization. Different amounts of FeCl3 aqueous solution (pH = 2.0) were added to the medium with the resultant concentrations of 10⁻⁵, 0.005, 0.05, 0.1, or 100 μM to generate iron-limited (FeCl3 =
5×10^5 to 0.1 μM) or iron-rich conditions (FeCl₃ = 100 μM). Iron-rich or iron-limited conditions were also generated using the Casamino Acid medium (CAA) or CAA medium + 20 mM aminothioether (Griffiths et al., 1981), in which single liter CAA medium consisted of Casamino Acid (BBI Life Sciences), 5.00 g; KH2PO4·3H2O, 1.18 g; MgSO4·7H2O, 0.25 g. Different amounts of tobramycin (0.0 to 4.0 μg·mL⁻¹) or gentamicin (2.0 μg·mL⁻¹) were added in the medium to adjust the antimicrobial stresses. We found that wild-type *P. aeruginosa* bacteria grow slowly in the CAA medium + 20 mM aminothioether during the first 12 h, reflecting iron limitation. However, growth became fast after 20 h of culturing and the bacterial culture eventually reached high density (OD₅₇₀: 10±0.5) in the presence of different conditions. Our results indicated that the CAA medium + 20 mM aminothioether is not suitable in generating an iron-limited condition. Therefore, the SSM medium was used in our evolutionary experiments.

**Construction of the *P. aeruginosa* mutants.** Deletion mutants ΔpvdA, ΔpvdAΔΔpvdA, ΔpvdAΔRST – opmQ were constructed by allelic exchange using a modified procedure for *P. aeruginosa*. We constructed unmarked deletion mutants by Fp-mediated excision of the antibiotic resistance marker β-lactamase. Firstly, two DNA fragments (from upstream and downstream gene) of 1.5 kb, respectively, were inserted into the one donor gene by PCR, which were cloned into a gene replacement vector pEX18AP via a three-piece ligation. Then the constructed plasmid was inserted into a gentamicin/tetracycline-resistant strain by electroporation. After the transformation, the recombinant strains were selected for colonies carrying the resistance marker. The deletion mutants were reportedly confirmed by PCR and sequencing. The constructed plasmids are listed in Supplementary Table 1. The PAO1 psfGFP, PAO1 ΔpsfGFP and PAO1 ΔpvdA ΔΔpvdAΔRST – opmQ strains were constructed by allelic exchange using a helper plasmid psfGFP strains were constructed by speci...
stress, intracellular concentration of PVDI and ferric concentration as below,

\[ P_1 = 1 - \exp \left( 1 - \frac{c_1 [PVDI]^0 + c_2 [PVDI]^0}{c_1 [PVDI]^1 + c_3} \right) \]  

\[ P_2 = 1 - \exp \left( 1 - \frac{c_1 [PVDI]^0 + c_2 [PVDI]^0}{c_1 [PVDI]^1 + c_3} \right) \]  

where the exponent \( \frac{[PVDI]^0}{c_1 [PVDI]^1 + c_3} \) reflects the fact that intracellular PVDI can allow bacteria to survive in the presence of environmental stress and Fe\(^{3+}\) would promote hydroxyl radical formation via Fenton reaction. \( \Delta t = 1\ \text{min} \).

We mix producer and non-producer strains to allow them to grow together in the presence of a given environmental stress, in which the dynamics of the system are assumed to exactly follow the differential equations as below,

\[ \frac{d[PVDI]^0}{dt} = P_t + \alpha [PVDI]^0 - y_t [PVDI]^1 - \mu_t [PVDI]^0, \]  

\[ \frac{d[PVDI]^1}{dt} = \alpha [PVDI]^0 - y_t [PVDI]^1 - \mu_t [PVDI]^1, \]  

\[ \frac{d[PVDI]^n}{dt} = \phi_1 y_{11} [PVDI]_{11}^1 + \phi_2 y_{12} [PVDI]_{12}^1 - (\phi_1 + \phi_2) a [PVDI]^0 - \delta [PVDI]^n, \]  

where \( \phi_1 \) and \( \phi_2 \) are the volume fractions (\( \phi \equiv V/V \)) of producer and non-producer strains, respectively, \( V \) and \( V \) are the bacterial and the system volumes, respectively, \( N \) is the bacterial number, and \( \delta \) is the global dilution rate in our evolutionary experiment. \( \phi_1 \) and \( \phi_2 \) are obtained by directly solving these differential equations using the iterative method with following initial conditions, \( \phi(0) = 0, [PVDI]^0(0) = [PVDI]_{11}^0 = [PVDI]_{12}^0 = 0 \) µM. The parameters used in our model are listed in Table 1.

To improve the evolutionary analysis, we evaluated the total population size (\( N(t) \)) of the invading strain during the competition. Note that \( N(t) \equiv \phi(t) V/V \). To ensure that \( N(t) \) (or \( \phi(t) \)) is independent of \( \delta \), we normalized \( N(t) \) by \( N(t)/N(t) \) to calculate a relative yield defined as below,

\[ \text{Relative yield def} = \log_{10} [N(t)/N(t)]. \]  

Data availability. All data generated or analysed during this study are available from the authors on reasonable request.

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Table 1 Parameters used for the theoretical modeling

| Parameter | Value |
|-----------|-------|
| \( \phi_i \) | 4 µM\(^{-1} \) |
| \( \alpha \) | 2.1 \times 10^8 \muM\(^{-2} \) |
| \( b_1 \) | 9250 (dimensionless) |
| \( b_2 \) | 3.74 (dimensionless) |
| \( b_3 \) | 0.42 (dimensionless) |
| \( b_4 \) | 2.64 (dimensionless) |
| \( E_{50} \) (405 nm laser) | 8 mW cm\(^{-2} \) |
| \( E_{50} \) (tobramycin) | 2.0 µg ml\(^{-1} \) |
| \( \gamma \) | 10\(^{-10} \) (dimensionless) |
| \( c_1 \) | 0.031 (dimensionless) |
| \( c_2 \) | 16.65 µM\(^{-1} \) |
| \( c_3 \) | 0.0013 (dimensionless) |
| \( [Fe^{3+}]_{\text{min}} \) | 10\(^{-5} \) µM |
| \( \alpha \) | 0.1 min\(^{-1} \) |
| \( \delta \) | 0.0085 min\(^{-1} \) |
| \( \rho \) | 0.018µg min\(^{-1} \) |

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Author contributions
FJ conceived the project. JHL, ZYJ performed the experiments. ZYJ performed theoretical calculations. ZYJ, AGX analyzed data. LN found that the photon-stresses can trigger the accumulation of PVDI. RRZ helped to construct bacterial strains. ZYJ and FJ contributed jointly to data interpretation and manuscript preparation. All authors reviewed the manuscript.

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