

Iron interferes with quorum sensing-mediated cooperation in *Pseudomonas aeruginosa* by affecting the expression of *ppyR* and *mexT*, in addition to *rhIR*[§]

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The stabilization of quorum sensing (QS) is vital for bacterial survival in various environments. Although the mechanisms of QS stabilization in certain conditions have been well studied, the impact of environmental factors has received much less attention. In this study, we show that the supplementation of 25 μ M iron in competition experiments and 50 μ M in evolution experiments to casein growth cultures significantly increased the possibility of population collapse by affecting elastase production. However, the expression of *lasI* and *lasR* remained constant regardless of iron concentration and hence this effect was not through interference with the LasIR circuit, which mainly regulates the secretion of elastase in *Pseudomonas aeruginosa*. However, the expression of *rhIR* was significantly inhibited by iron treatment, which could affect the production of elastase. Further, based on both reverse transcription quantitative polymerase chain reaction and gene knock-out assays, we show that iron inhibits the transcription of *ppyR* and enhances the expression of *mexT*, both of which decrease elastase production and correspondingly interfere with QS stabilization. Our findings show that environmental factors can affect the genes of QS circuits, interfering with QS stabilization. These findings are not only beneficial in understanding the mechanistic effect of iron on QS stabilization, but also demonstrate the complexity of QS stabilization by linking non-QS-related genes with QS traits.

Keywords: *Pseudomonas aeruginosa*, iron, quorum sensing, elastase, cooperation

Introduction

Quorum sensing (QS) is a cell-to-cell communication process

mediated by chemical signals (Whiteley *et al.*, 2017). Through this communication, bacteria produce a range of secreted factors, or “public goods,” that aid in ingesting nutrients, providing scaffolding for biofilm formation, and attacking host cells (Schuster *et al.*, 2013). QS is a form of bacterial cooperation as public goods produced by cells are also beneficial to neighboring cells (Dandekar *et al.*, 2012). However, the production of public goods is costly for individual cells and cooperation within a population is consequently at risk of being exploited by non-producing cheaters, which interfere with the stabilization of QS cooperation (Sandoz *et al.*, 2007).

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen found in a wide variety of environments. As a paradigm model organism for QS research, *P. aeruginosa* has been reported to have at least four well-known QS circuits: LasIR, RhIR, *Pseudomonas* quinolone signal (PQS), and integrated quorum sensing system (IQS). Three kinds of QS signals have been detected in *P. aeruginosa*: *N*-3-oxo-dodecanoyl homoserine lactone (3OC₁₂-HSL), *N*-butanoyl homoserine lactone (C₄-HSL), and 2-Heptyl-3-hydroxy-4(1H)-quinolone (Pearson *et al.*, 1994, 1995), which bind to LasIR, RhIR, and PQS respectively. Notably, 3OC₁₂-HSL can also bind to IQS as well as LasR (Lee *et al.*, 2013; Papenfort *et al.*, 2016). The detailed network in *P. aeruginosa* provides a good base for understanding the stabilization of QS in bacteria.

The stabilization of QS in constant conditions has been well studied. The mechanisms for cheater control have been described and include kin-selection (Diggle *et al.*, 2007), metabolic prudence (Xavier *et al.*, 2011), metabolic constraints (Dandekar *et al.*, 2012), and policing (Wang *et al.*, 2015). Although bacteria have evolved various strategies to maintain QS cooperation, destabilization of QS and the collapse of the population can occur, especially in the presence of different nitrogen sources (Wang *et al.*, 2017). As interference with QS stabilization could have potential applications in medicine or disinfectant development, there is currently great interest in understanding the effects and influence of environmental factors on this process.

Growth of *P. aeruginosa* with casein as the sole source of carbon and energy requires production of the QS-regulated protease elastase. Elastase digests casein into peptides and amino acids that can be taken up and used by *P. aeruginosa* cells regardless of whether they produce elastase themselves (Sandoz *et al.*, 2007). This culture medium has been frequently used to study the cooperation of QS. In previous studies, we focused on conditional QS cooperation in different nitrogen sources (Wang *et al.*, 2017; Yan *et al.*, 2018). In addition, the involvement of carbon metabolism in QS coop-

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eration has been explored in *Vibrio cholerae* (Hawver *et al.*, 2016). Besides nutrition, metal elements also appear to be important for bacterial activities. For example, iron is essential for a wide variety of biological functions, from DNA synthesis to electron transport (Zhang *et al.*, 2018b). However, the effect of iron on QS cooperation remains unknown, which limits our understanding of conditional QS and QS stabilization.

Here, we show that the supplementation of 25 μM iron to casein media is sufficient to interfere with QS stabilization. Interestingly, iron does not affect the QS circuit *LasR* by disturbing elastase production and the expression of *lasI* and *lasR* remained unchanged under different iron concentrations. Iron does, however, affect the expression of *ppyR* (PA-2663) and *mexT* (PA2492), decreasing the production of elastase thereby interfering with QS cooperation. Thus, we show that essential elements can affect the genes of QS circuits and interfere with QS stabilization, which not only helps us to understand the impact of environmental factors on QS cooperation but also contributes to our knowledge of the complexity of QS in nature.

Materials and Methods

Bacterial strains and growth conditions

The following *P. aeruginosa* strains were utilized: PAO1-UW (Stover *et al.*, 2000) and PAO1-UW-derived *lasR*, *ampR*, *ppyR*, *mexT*, *ppyRampR*, *ppyRmexT*, and *ampRmexT* mutants. We used homologous recombination to generate mutants as previously described (Sambrook *et al.*, 1989). Polymerase chain reaction (PCR)-amplified DNA fragments flanking *ppyR*, *ampR*, and *mexT* were cloned into pEXG2 (Rietsch *et al.*, 2005). Briefly, *Escherichia coli* S17-1 containing pEXG2 constructs were mated with PAO1 or PAO1 single mutants. Transconjugants were selected on *Pseudomonas* Isolation Agar (PIA) containing gentamicin. Deletion mutants were selected on PIA containing 5% (w/v) sucrose. Mutants were confirmed by PCR of genomic DNA. Primers used in this study are described in Supplementary data Table S1.

Bacteria were grown in either minimal medium with 1% (w/v) sodium caseinate as a carbon source (casein broth) (Dandekar *et al.*, 2012), 0.5% (w/v) casein ampicase acid (CAA broth), or in LB buffered with 20 mM *N*-morpholinopropanesulfonic acid (LB-MOPS) (Wang *et al.*, 2015); iron was absent from this minimal media. As required, 12.5, 25, 50, 100, and 200 μM Fe^{2+} (in the form of FeSO_4) was added to the minimal medium, which were filter sterilized using 0.22 μm membranes. Unless otherwise specified, cultures were grown in 16 mm test tubes containing 4 ml media, with shaking (225 rpm) at 37°C.

Phosphate buffered saline (PBS) was prepared by dissolving 8.006 g NaCl, 0.201 g KCl, 1.540 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.191 g KH_2PO_4 in 1 L DW and sterilized at 121°C for 20 min.

Short-term competition and long-term evolution experiments

Wild type (WT) *P. aeruginosa* and *ΔlasR* strains, each grown separately overnight in LB-MOPS, were combined at an initial cheater ratio of 1% and inoculated into casein broth containing 0–200 μM FeSO_4 . At 24 h intervals, 40–80 μl of culture were transferred to fresh casein broth with corresponding FeSO_4 supplementation. Additionally, either 0.1 mg/L of elastase or 50 mg/L of rhamnolipid were supplemented into cultures containing 100 μM of FeSO_4 as complementary experiments. On day seven (or earlier if there was no growth after transfer prior day seven), we determined the abundance of cheaters as described below. Five independent tubes were performed as replicates.

In parallel, the WT strain was cultured in LB-MOPS for 12 h after which 50 μl was transferred to casein broth containing 0–200 μM FeSO_4 . Fresh casein broth with corresponding FeSO_4 supplementation was inoculated with 40–80 μl of culture at 24 h intervals for 30 days. Five independent tubes were performed as replicates. As described in Dandekar *et al.* (2012), we determined the abundance of cheaters by plating cells on LB-MOPS agar and transferring isolated colonies to skim milk agar plates to enumerate *LasR*[−] mutant cheats in the population. WT strains produce elastase resulting in a zone of clearance on skim milk agar, while *LasR*[−] mutants do not. For each plate, we screened at least 100 colonies.

Table 1. Fate of *P. aeruginosa* population collapse under different iron treatments

Experiment	Iron (μM)	Replicates (N)	Collapses (N)	Ratio of collapse (n/N, %)	Days of collapse (Day)	Cheaters in stable population (%)
^a Short-term competition	0	5	0	0	NC ^c	22.50 ± 6.07
	12.5	5	0	0	NC	38.20 ± 4.52
	25	5	2	40	7	42.83 ± 5.41
	50	5	2	40	5	36.93 ± 6.21
	100	5	2	40	5	46.90 ± 10.20
^b Long-term evolution	200	5	3	60	5	42.03 ± 5.28
	0	5	0	0	NC	24.10 ± 8.76
	12.5	5	0	0	NC	26.78 ± 11.44
	25	5	0	0	NC	33.49 ± 11.68
	50	5	1	20	20	48.83 ± 12.64
100	5	1	20	16	48.10 ± 16.65	
200	5	1	20	15	49.57 ± 7.00	

^a Short-term competition: The initial frequency of cooperators was 99%, results are at 7 days.

^b Evolution: Start only with cooperators, results are at 30 days.

^c NC, No collapse occurred.

Analysis of the effect of iron on QS-related genes and products

Bacteria were cultured in the presence of different concentrations of Fe^{2+} . Once logarithmic growth had been reached, cells were harvested and analyzed for the expression of QS-related genes. Supernatants were used to analyze QS-related products, including elastase, and rhamnolipid.

The expression of QS-related genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsABCDE*, and *pqsR*) was analyzed using real-time reverse transcription PCR (RT-qPCR). Total RNA was extracted using the Trizol method (Rutherford *et al.*, 2011). DNA contamination was eliminated by DNase I treatment. cDNA was synthesized from total RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR was performed using a CFX96 RT-PCR detection system (Bio-Rad) and the SYBR Premix Ex Taq II kit (TaKaRa). Primers and annealing temperatures are listed in Table 1. The housekeeping gene *proC* was used as an endogenous control to normalize the data. Relative gene expression was determined using the comparative Ct ($2^{-\Delta\Delta C_t}$) method. Primers used in this study are described in Supplementary data Table S1.

The elastase content was measured using the Pierce Fluorescent Elastase Assay kit (Thermo Fisher Scientific). Briefly, the culture was centrifuged at 12,000 rpm for 15 min. Then, 100 μl of the supernatant was mixed with 100 μl of succinylated casein solution (1:500 mixture of 2 g/L lyophilized succinylated casein and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) working solution, pH = 8.5) and incubated for 45 min in the dark at room temperature. Fluorescence was detected at 450 nm using a plate reader (SpectraMax[®] i3).

Rhamnolipid was quantified according to a previously published method (Welsh and Blackwell, 2016) but with modifications. Briefly, 1 ml of culture was adjusted to pH 2.0 with 1 mmol/L HCl. The culture was then extracted using a mixture of chloroform and methanol (v/v = 2:1). The organic phase was collected, mixed with 1.8 ml of 0.19% (w/v) orcin and 50% (v/v) H_2SO_4 , and allowed to incubate at 80°C for 30 min. Finally, the absorbance of the mixture was measured

at 421 nm.

Statistical analysis

All data are presented as the mean \pm standard deviation (SD). Differences between groups were identified by analysis of variance using SPSS (version 22). Differences were considered significant when $p < 0.05$.

Results

Iron interferes with QS stabilization in bacterial populations

Iron is essential for a wide variety of biological functions, from DNA synthesis to electron transport (Oglesby *et al.*, 2008). We were curious about the effect of iron on the stabilization of QS, as QS is mainly regulated by a series of social behaviors (Abisado *et al.*, 2018). Here, different concentrations of iron (0–200 μM) were added to casein broth to investigate its effect on QS stabilization in populations of PAO1. As shown in Table 1, in short-term competition experiments, the ratio of population collapse with 25–200 μM iron treatments was 40–60%, which was significantly higher than treatments with 0–12.5 μM iron, where no population collapse was observed. In addition, the rate of population collapse correlated positively with the concentration of iron.

The proportion of 1% cheaters used in this study is relatively high for a natural setting, as cheaters will naturally occur as the population develops (Özkaya *et al.*, 2018). To assess the effect of iron on population stability with an evolving number of cheaters, a long-term evolutionary experiment was performed. The trend of QS stabilization was similar to the short-term competition experiment. However, because of a series of mechanisms that inhibit rapid increases in the proportion of cheaters (Wilder *et al.*, 2011; Xavier *et al.*, 2011; Wang *et al.*, 2015; Abisado *et al.*, 2018), the probability of population collapse was lower than the short-term competition experiment. No population collapse was observed with 0–25 μM

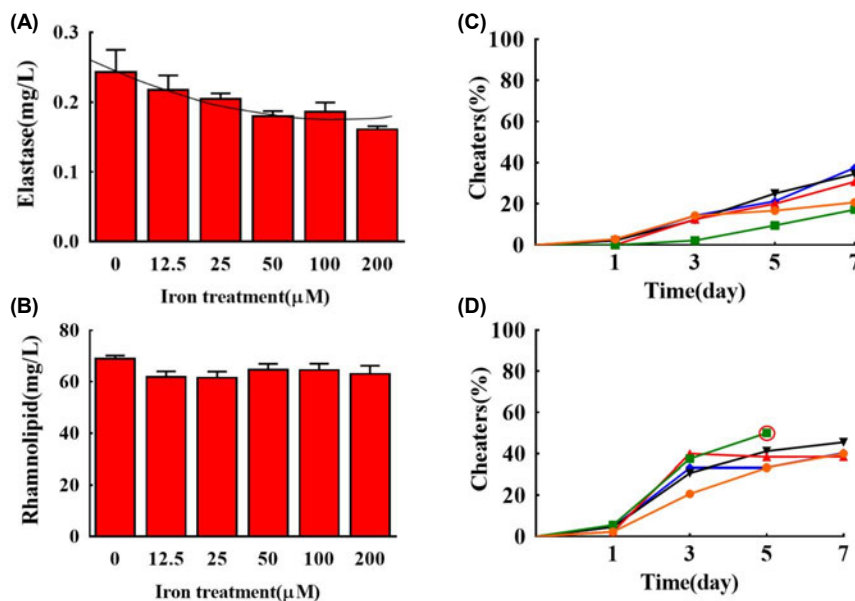


Fig. 1. Effect of iron on public goods and population stabilization. Effect of iron on (A) elastase and (B) rhamnolipid production in *P. aeruginosa*. All experiments were independently repeated thrice. Competition between cooperators and cheaters in the presence of (C) 0.1 mg/L elastase and 100 μM iron treatment and (D) 50 mg/L rhamnolipid and 100 μM iron treatment. The initial frequency of cooperators was 99%. Circles indicate population collapse in the competition experiment. Each bar in (A) and (B) represents the average of parallel repetitions. In (C) and (D), each line represents individual replicates and five parallel repetitions are represented by five different colors, performed under identical conditions.

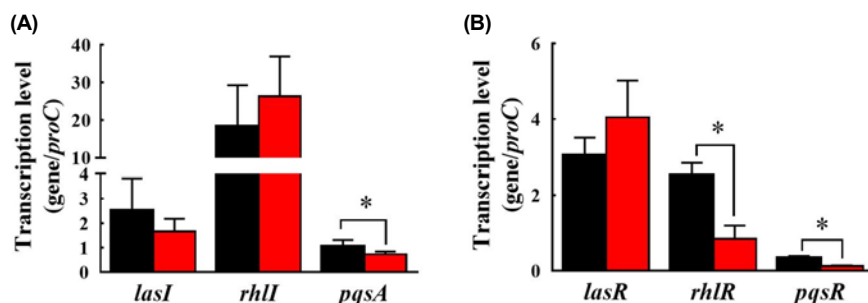


Fig. 2. Effect of iron on LasIR, RhIR, and PQS circuits in *P. aeruginosa*. Gene expression of (A) QS signals and (B) QS receptors. Black bars, without iron treatment; red bars, 100 μ M iron treatment.

iron treatment, while 20% population collapse occurred with 50–200 μ M iron treatment. The frequency of cheaters at day 30 increased with the concentration of iron, from 24% with no iron treatment to 50% with 200 μ M iron.

As QS is a cell-density dependent regulation system, the effect of iron on bacterial growth was assessed. There was no significant difference in the growth of bacteria under the iron treatment conditions of 0 and 100 μ M (Supplementary data Fig. S1). To mitigate the effect of pH variability on iron bioavailability, the pH of different treatments were measured. Due to the presence of PBS, the pH of cultures with different iron treatments were stable at 7.0 (Supplementary data Fig. S2). Taken together, these results indicate that the addition of iron interferes with the stabilization of QS without inhibition of growth. The low frequency of collapsed populations in the long-term evolution experiment can be partly attributed to the mechanisms for cheater control, such as policing.

Elastase shortage contributes to instability of QS but not via the LasIR circuit

As cheaters do not secrete and rely on public goods, a sufficient supply is the bases for bacterial population stability (Diggle *et al.*, 2007). In order to explore the reasons for the destabilization of QS observed in the above experiments, we assessed the effect of iron on the production of public goods. As shown in Fig. 1A, incremental increase of iron in the culture media caused the production of elastase to decrease significantly. Although there was no difference in elastase concentration with 0–12.5 μ M iron treatments ($p > 0.05$), elastase production decreased by 15.9–33.8% with 25–200 μ M iron treatments. The concentration of rhamnolipid also decreased after iron supplementation, although no differences were observed with 12.5–200 μ M iron treatments ($p > 0.05$) (Fig. 1B).

As the amount of public goods determines QS stabilization, we added elastase and rhamnolipid into the cultures treated with 100 μ M iron to assess the possibility of arresting population collapse by increasing available public goods. All five groups with the addition of 0.1 mg/L elastase were stable (Fig. 1C) while one group with the addition of 50 mg/L rhamnolipid collapsed (Fig. 1D). In casein media, elastase is required for *P. aeruginosa* to digest proteins into amino acids for metabolism (Dandekar *et al.*, 2012). It is therefore possible that the addition of iron to casein broth affects the production of elastase but not rhamnolipid, thereby interfering with QS stabilization. To test this hypothesis, we assessed the expression of the corresponding genes.

It has been reported that the genes of the IQS circuit (Amb-BCDE-IqsR) are not related to elastase production (Lee *et al.*, 2013; Papenfort *et al.*, 2016) and were therefore not analyzed here. We analyzed the effect of iron on the expression of *lasI*, *lasR*, *rhII*, *rhIR*, *pqsA*, and *pqsR*. As shown in Fig. 2, the addition of 100 μ M iron did not affect the expression of *lasI* or *lasR*. The expression of *rhIR* and *pqsR* decreased with 100 μ M iron treatment when compared to samples with no iron treatment and could explain the observed reduction of rhamnolipid (Fig. 1B). However, as no decrease in the expression of *lasI* and *lasR* were observed, the reduction of elastase (Fig. 1A) could not be attributed to iron-mediated interference on LasIR circuit, which has been reported to mainly regulate elastase production (Schuster *et al.*, 2007). Although *rhIR* has a partial effect on the reduction of elastase, the effect is much lower than that of the *las* system (Whi-

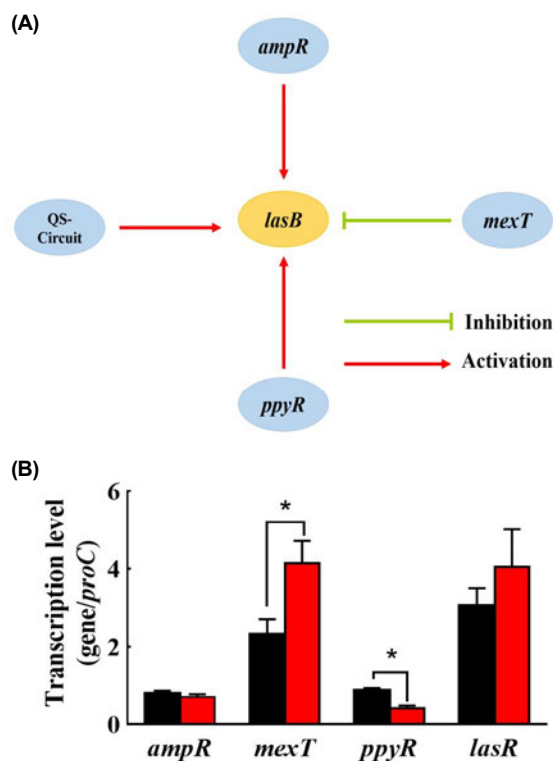


Fig. 3. Effect of iron on QS circuit gene expression in *P. aeruginosa*. (A) Genes that regulate the expression of *lasB*. (B) Effect of iron on the expression of *ampR*, *mexT*, *ppvR*, and *lasR*. Black bars, without iron treatment; red bars, 100 μ M iron treatment.

teley *et al.*, 1999). The reduction of rhamnolipids was not significant, indicating that iron has some down-regulatory effect on the *rhlR* gene, but the effect of iron on regulators of the RhlIR system is not clear. Thus, the mechanism by which iron interferes with elastase production needs to be explored further.

Both *ppyR* and *mexT* participate in the iron-mediated interference for elastase production

Through the Kyoto Encyclopedia of Genes and Genomes (<https://www.genome.jp/kegg/>), we found that in addition to *lasR*, three other genes—*ppyR*(PA2663), *ampR*(PA4109), and *mexT*(PA2492)—participate in the regulation of elastase. Both *ppyR* and *ampR* up-regulate the expression of elastase gene *lasB*, while *mexT* down-regulates its expression (Fig. 3A). Thus, we compared transcription of these genes in the absence or presence of 100 μ M iron. As shown in Fig. 3B, there were no differences in the expression of *ampR* and *lasR* with either 0 or 100 μ M iron treatment. The expression of *mexT* increased significantly with 100 μ M iron treatment when compared to no iron treatment ($p < 0.05$). Furthermore, the addition of 100 μ M of iron significantly decreased the expression of *ppyR* ($p < 0.05$). This indicates that iron does not regulate the production of elastase by affecting *lasR* and *ampR* but instead does so by affecting the expression of *mexT* and *ppyR*. To confirm this, we constructed $\Delta ampR$, $\Delta ppyR$, and $\Delta mexT$ mutants. Neither $\Delta ampR$ nor $\Delta ppyR$ were able to grow in casein medium, suggesting that these genes may be important for elastase production. For the $\Delta mexT$ strain, the elastase content increased significantly, indicating that *mexT* has a strong inhibitory effect on elastase production. As the concentration of iron was increased, the production of elastase by $\Delta mexT$ decreased, suggesting that iron may decrease protease production by affecting *ampR* or *ppyR*.

Since $\Delta ampR$ and $\Delta ppyR$ showed no growth, we constructed $\Delta ampR\Delta ppyR$, $\Delta ampR\Delta mexT$, and $\Delta ppyR\Delta mexT$ mutants. As shown in Fig. 4A, $\Delta ampR\Delta ppyR$ was not able to grow in casein medium. However, as shown in Fig. 4B, with the removal of the inhibitory effect of *mexT*, $\Delta ampR\Delta mexT$, and $\Delta ppyR\Delta mexT$ were able to grow normally. Furthermore, with increasing concentrations of iron, the production of elastase

in $\Delta ampR\Delta mexT$ decreased while no significant difference in the production of elastase in $\Delta ppyR\Delta mexT$ was seen, suggesting that iron has an effect on *ppyR* but not *ampR*. As iron does not affect *LasR* expression, the effect of iron on elastase production in $\Delta ampR\Delta ppyR$, $\Delta ampR\Delta mexT$, and $\Delta ppyR\Delta mexT$ could be through *mexT* and *ppyR*. Combined with the results of qPCR and the lack of growth of the $\Delta ampR$, $\Delta ppyR$, and $\Delta ampR\Delta ppyR$ mutants, these findings show that iron mainly affects *MexT* regulation. Furthermore, the difference between $\Delta ampR\Delta mexT$ and $\Delta ppyR\Delta mexT$ shows that iron affects *ppyR* but not *ampR*.

Discussion

Bacterial QS coordinates a range of behaviors at the population level (Papenfort *et al.*, 2016). Although QS circuits are relatively conserved, the collapse of populations can happen due to mutations of QS-related genes and a shortage of public goods (Diggle *et al.*, 2007; Schuster *et al.*, 2013). Thus, the stabilization of QS has gained attention with regards to better treatments for pathogenic infections and in the development of pollutant bioremediation (Shrout and Nerenberg, 2012; Curran *et al.*, 2018).

We are interested in the effects of environmental cues on the stabilization of bacterial QS. Previous studies have shown that ammonia can enhance the activity of the RhlIR system and the production of hydrogen cyanide, resulting in improvement of the efficiency of policing control (Yan *et al.*, 2018) and that engineered nanoparticles can affect bacterial QS under short-term exposure, while interference is restored after long-term exposure due to the adaptive evolution (Zhang *et al.*, 2018a). Here, we found that iron can interfere with QS-mediated cooperation (Table 1). Although there was no clear relationship between the dose of iron and destabilization of QS, the addition of more than 25 μ M iron in competition experiments and 50 μ M iron in evolution experiments significantly increased the possibility of population collapse. The influence of various environmental factors on QS stabilization not only suggests that bacterial QS is highly complicated and intelligent, but also a reminder that simple manipulation

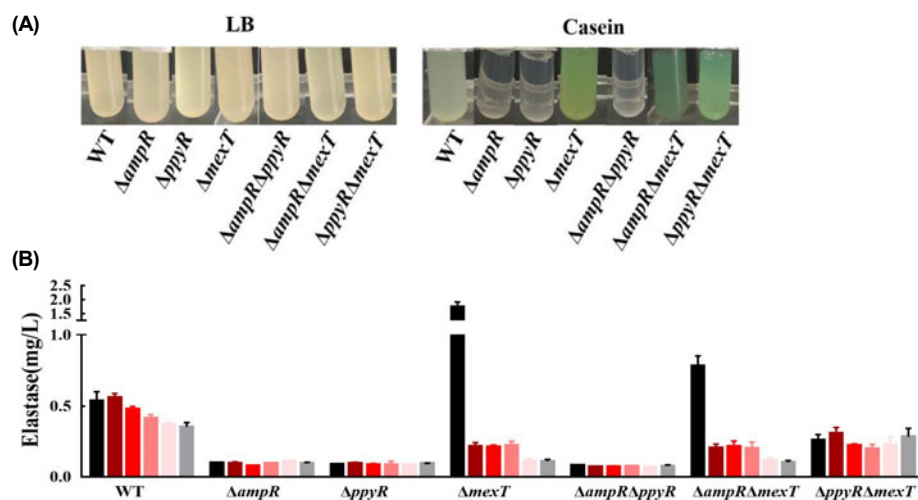


Fig. 4. Effect of iron on the expression of *LasB*-regulated genes in *P. aeruginosa*. (A) Growth of different mutants in both LB and casein media. (B) Effect of iron on elastase production in $\Delta ampR$, $\Delta ppyR$, $\Delta mexT$, $\Delta ampR\Delta ppyR$, $\Delta ampR\Delta mexT$, and $\Delta ppyR\Delta mexT$ mutants. From left to right: 0, 12.5, 25, 50, 100, and 200 μ M FeSO₄.

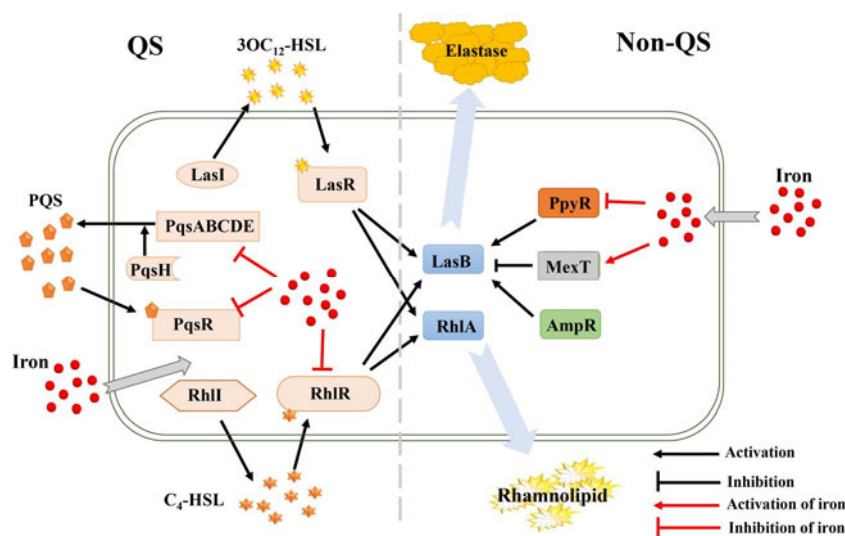


Fig. 5. Molecular mechanisms of the effects of iron on *P. aeruginosa*. Left and right pictures show the influence of iron on QS and non-QS processes, respectively.

of QS is not sufficient for its application.

It is well known that various public goods are regulated by QS. In *P. aeruginosa*, QS signals, in addition to elastase, rhamnolipid, and siderophores, have been reported as public goods (Mund *et al.*, 2017). Currently, little is known about how substances from QS interact with each other, whether directly or indirectly (Brown and Taylor, 2010). Previously, PQS has been shown to cause iron starvation, leading to increased production of costly siderophores and relative fitness of siderophore cheats (Popat *et al.*, 2017). Here, we show that iron can directly affect QS-mediated cooperation by interfering with the public good elastase. Although iron has a dose-dependent relationship with PQS-related QS stabilization, a similar relationship was not seen with LasIR-related QS stabilization.

Iron can be chelated by PQS, one of the QS signal molecules in *P. aeruginosa* (Bredenbruch *et al.*, 2006), demonstrating a direct connection between iron and the PQS circuit. In this study, we found that iron can interfere with QS cooperation in a manner independent from the PQS circuit and dependent on the supply of *lasB*-encoded elastase (Fig. 5). We also found that iron did not affect the expression of *lasI* and *lasR*, which mainly regulate the production of elastase. Because the effect of *rhl* system is much lower than that of the *las* system (Whiteley *et al.*, 1999). We need to further explore the mechanism of iron on proteases. Finally, we found that iron regulates the production of elastase and interferes with QS cooperation by affecting the expression of *mexT* and *ppyR* (Fig. 5). As neither *mexT* nor *ppyR* are under any QS circuits, this shows an integration of environmental cues in the stabilization of QS via non-QS-related genes.

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Author Contributions

F.S., L.W., H.F., D.S., and M.W. designed research; F.S., N.L., and L.W. performed research; F.S., L.W., H.F., and M.W. analyzed data; and F.S., and L.W. wrote the paper.

Conflict of Interest

The authors declare no competing interests.

References

- Abisado, R.G., Benomar, S., Klaus, J.R., Dandekar, A.A., and Chandler, J.R. 2018. Bacterial quorum sensing and microbial community interactions. *mBio* 9, e02331-17.
- Bredenbruch, F., Geffers, R., Nimtz, M., Buer, J., and Häussler, S. 2006. The *Pseudomonas aeruginosa* quinolone signal (PQS) has an iron-chelating activity. *Environ. Microbiol.* 8, 1318–1329.
- Brown, S.P. and Taylor, P.D. 2010. Joint evolution of multiple social traits: a kin selection analysis. *Proc. R. Soc. B.* 277, 415–422.
- Curran, C.S., Bolig, T., and Torabi-Parizi, P. 2018. Mechanisms and targeted therapies for *Pseudomonas aeruginosa* lung infection. *Am. J. Respir. Crit. Care Med.* 197, 708–727.
- Dandekar, A.A., Chugani, S., and Greenberg, E.P. 2012. Bacterial quorum sensing and metabolic incentives to cooperate. *Science* 338, 264–266.
- Diggle, S.P., Griffin, A.S., Campbell, G.S., and West, S.A. 2007. Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450, 411–414.
- Hawver, L.A., Giulietti, J.M., Baleja, J.D., and Ng, W.L. 2016. Quorum sensing coordinates cooperative expression of pyruvate metabolism genes to maintain a sustainable environment for population stability. *mBio* 7, e01863-16.
- Lee, J., Wu, J., Deng, Y., Wang, J., Wang, C., Wang, J., Chang, C., Dong, Y., Williams, P., and Zhang, L.H. 2013. A cell-cell communication

- signal integrates quorum sensing and stress response. *Nat. Chem. Biol.* **9**, 339–343.
- Mund, A., Diggle, S.P., and Harrison, F. 2017. The fitness of *Pseudomonas aeruginosa* quorum sensing signal cheats is influenced by the diffusivity of the environment. *mBio* **8**, e00353-17.
- Oglesby, A.G., Farrow, J.M., Lee, J.H., Tomaras, A.P., Greenberg, E.P., Pesci, E.C., and Vasil, M.L. 2008. The influence of iron on *Pseudomonas aeruginosa* physiology a regulatory link between iron and quorum sensing. *J. Biol. Chem.* **283**, 15558–15567.
- Özkaya, Ö., Balbontín, R., Gordo, I., and Xavier, K.B. 2018. Cheating on cheaters stabilizes cooperation in *Pseudomonas aeruginosa*. *Curr. Biol.* **28**, 2070–2080.
- Papenfort, K. and Bassler, B.L. 2016. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat. Rev. Microbiol.* **14**, 576–588.
- Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., and Greenberg, E.P. 1994. Structure of the auto-inducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91**, 197–201.
- Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92**, 1490–1494.
- Popat, R., Harrison, F., da Silva Ana, C., Easton Scott, A.S., McNally, L., Williams, P., and Diggle, S.P. 2017. Environmental modification via a quorum sensing molecule influences the social landscape of siderophore production. *Proc. R. Soc. B.* **284**, 20170200.
- Rietsch, A., Vallet-Gely, I., Dove, S.L., and Mekalanos, J.J. 2005. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **102**, 8006–8011.
- Rutherford, S.T., van Kessel, J.C., Shao, Y., and Bassler, B.L. 2011. AphA and LuxR/HapR reciprocally control quorum sensing in *Vibrios*. *Genes Dev.* **25**, 397–408.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York, USA.
- Sandoz, K.M., Mitzimberg, S.M., and Schuster, M. 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc. Natl. Acad. Sci. USA* **104**, 15876–15881.
- Schuster, M. and Greenberg, E.P. 2007. Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics* **8**, 287.
- Schuster, M., Sexton, D.J., Diggle, S.P., and Greenberg, E.P. 2013. Acyl-homoserine lactone quorum sensing: from evolution to application. *Annu. Rev. Microbiol.* **67**, 43–63.
- Shrout, J.D. and Nerenberg, R. 2012. Monitoring bacterial twitter: does quorum sensing determine the behavior of water and wastewater treatment biofilms? *Environ. Sci. Technol.* **46**, 1995–2005.
- Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warren, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., et al. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964.
- Wang, M.Z., Lai, B.M., Dandekar, A.A., Yang, Y.S., Li, N., Yin, J., and Shen, D.S. 2017. Nitrogen source stabilization of quorum sensing in the *Pseudomonas aeruginosa* bioaugmentation strain SD-1. *Appl. Environ. Microbiol.* **83**, e00870-17.
- Wang, M.Z., Schaefer, A.L., Dandekar, A.A., and Greenberg, E.P. 2015. Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. *Proc. Natl. Acad. Sci. USA* **112**, 2187–2191.
- Welsh, M. and Blackwell, H. 2016. Chemical genetics reveals environment-specific roles for quorum sensing circuits in *Pseudomonas aeruginosa*. *Cell Chem. Biol.* **23**, 361–369.
- Whiteley, M., Diggle, S.P., and Greenberg, E.P. 2017. Progress in and promise of bacterial quorum sensing research. *Nature* **551**, 313–320.
- Whiteley, M., Lee, K.M., and Greenberg, E.P. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **96**, 13904–13909.
- Wilder, C.N., Diggle, S.P., and Schuster, M. 2011. Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the *las*, *rhl* and *pqs* quorum-sensing systems. *ISME J.* **5**, 1332–1343.
- Xavier, J.B., Kim, W., and Foster, K.R. 2011. A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **79**, 166–179.
- Yan, H.C., Wang, M.Z., Sun, F., Dandekar, A.A., Shen, D.S., and Li, N. 2018. A metabolic trade-off modulates policing of social cheaters in populations of *Pseudomonas aeruginosa*. *Front. Microbiol.* **9**, 337.
- Zhang, Y., Gao, J., Wang, L., Liu, S., Bai, Z., Zhuang, X., and Zhuang, G. 2018a. Environmental adaptability and quorum sensing: iron uptake regulation during biofilm formation by *Paracoccus denitrificans*. *Appl. Environ. Microbiol.* **84**, e00865-18.
- Zhang, Y.Y., Li, N., Wang, M.Z., Feng, H.J., Xu, C., and Xu, F. 2018b. Interference of non-lethal levels of graphene oxide in biofilm formation and adaptive response of quorum sensing in bacteria. *Environ. Sci.: Nano* **5**, 2809–2818.