Nutrient reduction induced stringent responses promote bacterial quorum-sensing divergence for population fitness

Kelei Zhao1,2, Xikun Zhou2, Wujiao Li2, Xiuyue Zhang1,3 & Bisong Yue3

Bacteria use a cell-cell communication system termed quorum-sensing (QS) to adjust population size by coordinating the costly but beneficial cooperative behaviors. It has long been suggested that bacterial social conflict for expensive extracellular products may drive QS divergence and cause the “tragedy of the commons”. However, the underlying molecular mechanism of social divergence and its evolutionary consequences for the bacterial ecology still remain largely unknown. By using the model bacterium Pseudomonas aeruginosa PAO1, here we show that nutrient reduction can promote QS divergence for population fitness during evolution but requiring adequate cell density. Mechanically, decreased nutrient supplies can induce RpoS-directed stringent response and enhance the selection pressure on lasR gene, and lasR mutants are evolved in association with the DNA mismatch repair “switch-off”. The lasR mutants have higher relative fitness than QS-intact individuals due to their energy-saving characteristic under nutrient decreased condition. Furthermore an optimal incorporation of lasR mutants is capable of maximizing the fitness of entire population during in vitro culture and the colonization in mouse lung. Consequently, rather than worsen the population health, QS-coordinated social divergence is an elaborate evolutionary strategy that renders the entire bacterial population more fit in tough times.

Bacterial individuals communicate by using small, diffusible signal molecules in a process termed quorum-sensing (QS) for population fitness in a density-dependent manner1. The introduction of the concept QS has significantly enhanced the role of cooperative behaviors during bacterial social activities2,3. In a healthy cooperation system, the QS-intact individuals (cooperators) synthesize costly extracellular products (public goods) such as proteases, signal molecules and other chemical components with important biological activity for the fitness whole population4. However, such cooperation was considered unstable and vulnerable to invasion by the QS-deficient individuals (cheaters) who do not produce but still consume those public goods from others cooperating. Therefore, the current paradigm in sociomicrobiology is that increased frequency of QS-deficiency may compromise the fitness of entire population3–6.

Pseudomonas aeruginosa is a formidable Gram-negative opportunistic pathogen with large genome size normally causes acute or chronic infections in individuals who are burn patients, undergoing cancer chemotherapy, trauma, and especially those with genetic disease cystic fibrosis (CF)7. It is well-known that the QS system of P. aeruginosa is dominated by the central regulator LasR and triggered by the signal molecule, N-(3-oxododecanoyl)-HSL (3OC12-HSL)8–10. The lasR mutants with impaired QS function were defined as typical cheaters11. Clinical evidence showed that the P. aeruginosa lasR mutants were commonly identified from the lungs of CF patients who suffered a long-term pseudomonal infection12,13, indicating that social cheating can naturally happen in P. aeruginosa population during chronic lung infection.

Although several ingenious experiments have investigated the de novo emergence of lasR mutant in QS-required or rich medium6,11,14,15, the underlying molecular mechanism and its effect on population fitness still

1Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610064, China. 2State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu 610064, China. 3Sichuan Key Laboratory of Conservation Biology on Endangered Wildlife, College of Life Science, Sichuan University, Chengdu 610064, China. Correspondence and requests for materials should be addressed to X.Z. (email: zhangxy317@126.com) or B.Y. (email: bsyue@scu.edu.cn)
remain largely unknown. And these analyses may have neglected the role of nutrient supply in the development of social behaviors. Both bacterial proliferation and cooperation are energy cost, and the proportion of energy allocation into these behaviors may directly determine the fitness of population. In a specific environment unit with less resource available, bacteria are likely to raise the investment into cooperation. However, when the environmental nutrient level is insufficient to afford the intense QS with high cell density, the social conflict for public goods may promote the divergence of QS. Previous mathematical modeling also suggested that food is more efficiently used when there is a famine. Moreover, starvation is considered as the major signal guiding bacteria entry into stationary phase, and the altered global gene expression pattern and morphological change in this phase will increase bacterial viability against various environmental pressures. Therefore, the emergence of QS-deficient individuals is probably approved by the population in response to nutrient reduction for further fitness.

To test our hypothesis, in this study we first perform a series of in vitro evolution of \( P. \) aeruginosa PAO1 in QS medium and rich medium with gradient nutrient levels. We find that nutrient reduction can promote bacterial social divergence but requiring adequate cell density. And this process is modulated by RpoS-dominated stringent response with the association of suppressed DNA mismatch repair (DMMR) system, as determined by the transcriptome analysis. We have also experimentally confirmed that \( \text{lasR} \) mutant has higher relative fitness than QS-proficient wild type (WT) PAO1 by escaping from the metabolic burden, and the incorporation of \( \text{lasR} \) mutants at optimal proportion can improve the fitness of entire population under nutrient decreased conditions and in the lungs of acutely and chronically infected mouse models.

Results

Nutrient reduction promotes bacterial social divergence during evolution. To verify the idea that nutrient reduction may serve as selection pressure and promote the de novo emergence of QS-deficient individuals, we performed a series of long-term evolution of WT PAO1 in QS-required and rich medium containing gradient concentrations of nutrient levels.

In vitro evolution in QS medium. Most of the previous evolutionary screenings of \( \text{lasR} \) mutants were performed in QS medium, and they concluded that \( \text{lasR} \) mutants can emerge under culture conditions that require QS-dependent extracellular products for growth. In this study, we further extended this conclusion by culturing WT PAO1 in M9 medium containing gradient concentrations of caseinate with or without pre-added 3OC12-HSL, to investigate the relationship between nutrient supply and QS divergence. In M9-caseinate medium, caseinate as the sole carbon source should be broken down by the QS-dominated elastase (encoded by \( \text{lasB} \) gene) before use. WT PAO1 can grow in this medium by developing QS, whereas \( \text{lasR} \) mutants can only grow by filching the elastase produced by WT PAO1. Previous studies demonstrated that exogenous QS signal could increase elastase production, and this suggested that the pre-added QS signal may also contribute to QS divergence under nutrient reduction conditions.
As shown in Fig. 1a, QS-deficient strains were first isolated from the culture with 0.3% caseinate on day 8, followed by from 0.75% and 1% caseinate medium on day 12. This result suggested that the time duration of QS divergence can be shortened with appropriately decreased nutrient supplies during evolution, as our screening and previous reports showed that lasR mutant would emerge after 10–15 days in 1% caseinate medium$^{6,11,21}$. Moreover, the pre-addition of QS signal could elevate the proportions of QS-deficient strains among each culture. The lasR genes of a total of 50 adenosine growth-negative isolates were sequenced, finally, 3 kinds of point mutations and 1 truncation in transcription initiation region (lasR1, lasR2, lasR3, and lasR4) were identified and all the isolates had a LasR-null phenotype (Supplementary Table 1).

Surprisingly, QS-deficient strains of 0.25% and 0.1% caseinate medium with pre-added signal were identified on day 16 and 24, and that from the culture without pre-added signal were even later. We speculated that this might because the development of QS is cell density dependent (Fig. 1b). The low cell density caused by insufficient nutrient supplies would decrease QS activity$^4$, and therefore restricted QS divergence. This is also accordant to the fact that there are scarce lasR mutants isolated from natural environments. The fractions of QS-deficient strains increased as culturing progressed and finally reached a stable equilibrium with the QS-intact counterparts, and the highest final proportion of QS-deficient strains was observed in 0.1% caseinate medium, followed by 0.25%, 0.5%, 0.75% and 1% culture (Fig. 1a). A recent study suggested that this equilibrium is dominated by a sort of “policing”, where the QS-intact individuals will use the second QS system, the Rhl system, to constrict QS-deficient individuals increasing by producing toxic cyanide$^{21}$.

In vitro evolution in rich medium. Although rich medium were considered as having equal selective advantage on QS-intact and -deficient individuals$^{14,25}$, we were attracted by the result that lasR mutant reached a slightly lower maximal cell density than WT PAO1$^{14}$, this indicated that a proficient QS system may also benefit population fitness in rich medium. We then repeated the in vitro evolution assay by culturing WT PAO1 in gradient dilutions of 3-(N-morpholino)-propanesulfonic acid (MOPS, pH 7.0)-buffered (to eliminate the effect of pH variation) LB broth (rich medium). This experiment was also repeated in a biofilm-related static culture, because biofilms are considered as efficient defense system that separating the bacterial population into units, and multiple lines of evidences had shown that lasR mutants are often isolated from biofilms in chronically infected patients with CF$^{22,23}$.

When WT PAO1 was cultured in gradient dilutions of LB broth, although the growth rates and final cell densities were varied, the stationary phases were observed after 20 h (Supplementary Fig. 1). And we surprisingly noticed that the liquid color of 1/2-strength LB became visibly green compared with that of other dilutions after the 1 cycle (Supplementary Fig. 2), indicating a hypervariable QS system at this nutrient level and as confirmed by measuring the expression of lasB gene (Supplementary Fig. 3). These interesting findings suggested that the social cooperation in rich medium can be magnified by appropriately reducing the nutrient level. Accordingly, QS-deficient strains of shaking cultures were first identified from the 1/2-strength LB broth without pre-added QS signal on day 21, followed by 1/4-strength LB broth on day 28. No lasR mutant was identified in full-strength LB. When QS signal was pre-added, lasR mutants were also first identified from the 1/2-strength LB broth on day 14, followed by 1/4- and full-strength LB broth on day 21 and 28, respectively (Fig. 1c). For the biofilm-related selection, similar time points of lasR mutant emergences as in shaking culture were observed (Fig. 1d). After sequencing of lasR genes in 50 putative lasR mutants, 3 kinds of point mutation and 1 truncation in transcription initiation region (lasR5, lasR6, lasR7, and lasR8) were identified and all the isolates had a LasR-null phenotype (Supplementary Tables 2 and 3). Thus, these findings confirmed that the evolvement of bacterial social divergence can be promoted under nutrient reduced conditions but requiring adequate cell density to guarantee the successful development of QS.

RpoS-dominated stringent response and DMMR “switch-off” contribute to social divergence. To reveal the underlying molecular mechanism of bacterial social divergence, we then analyzed the global expression profiles of WT PAO1 under different nutrient levels. We also chose gradient dilutions of LB broth, because the expression of QS-related genes might be significantly changed in QS medium$^{11}$. Total RNA of each culture was isolated at stationary phase and then conducted for RNA-Sequencing followed by differential expression analysis (Fig. 2a–c). Notably, cells grown in 1/2-strength LB broth showed elevated expression levels of QS-inducible genes and several exoproducts (Fig. 2d and Supplementary Table 4). This was also accordant to the visibly green color of 1/2-strength LB culture. DinB expression was significantly increased in 1/2-strength culture ($P < 0.01$), and the expression of MutS was decreased ($P < 0.001$) (Fig. 3a). When the rpoS gene was knocked out, the expression levels of lasR, lasB and dinB were significantly decreased ($P < 0.05$) (Fig. 3b), and these results were also accordant to the previous findings that LasR is positively regulated by RpoS$^{25}$. RpoS triggered lasR expression may contribute to explore potential nutrient factors by producing several exoproteins$^{20}$. To confirm the roles of RpoS and DMMR system in the evolvement of QS divergence, we then performed a long-term culture of PAO1-$\Delta$ rpoS, PAO1-$\Delta$ dinB, and PAO1-$\Delta$ mutS in M9 medium containing 0.5% caseinate. As expected, comparing with WT PAO1, the lasR gene was more error-prone in mutS mutant but more conserved in dinB mutant and rpoS mutant.
strains (Fig. 4). Therefore, our results here confirmed that nutrient reduction induced RpoS-dominated stringent response and DMMR “switch-off” jointly promote the mutagenesis of lasR gene.

LasR mutants consume less energy but more adapt to nutrient decreased condition. We then attempted to investigate why the lasR mutants can be frequently isolated from stressed P. aeruginosa population. The emergence of lasR mutants during evolution suggested that an intense QS system may not always be favored, especially when the population is undergoing nutrient reduction and insufficient to support continually increased social cooperation. The lasR mutant with impaired QS system may have higher relative fitness than QS-intact WT individuals by escaping from metabolic burden but benefit from others cooperating under the conditions with decreasing nutrient supplies.
We tested this hypothesis by co-culturing WT PAO1 and the lasR mutant counterpart (99:1) in gradient QS medium. As shown in Fig. 5a–e, the proportions of WT PAO1 and lasR mutant showed a convergent trend with time, and this trend could be improved by pre-addition of QS signal and decreasing caseinate supplies. The final proportion of lasR mutant became higher than WT PAO1 (P < 0.05) when the concentrations of caseinate were <0.25% (wt/v) (Fig. 5f). The relative fitness of lasR mutants was increased along with decreasing nutrient levels and the value (\(v\)) became >1.0 when caseinate levels were <0.25% (Supplementary Fig. 4). This indicated that the relative growth rate of lasR mutants is lower than WT PAO1 in high nutrient levels but faster when resource is decreasing. Subsequently, the total ATP productions of WT PAO1 and lasR mutant in the culture with gradient nutrient levels were measured to investigate the underlying mechanism of this growth divergence. Because lasR mutant cannot grow in QS medium without QS-intact counterpart, casamino acids (CAA, the product of caseinate digested by elastase) were used instead of caseinate as the sole carbon source. Theoretically, if WT PAO1 showed higher ATP production level than lasR mutant in M9-CAA medium, the ATP production of WT PAO1 individual should be much higher than in lasR mutant when they were co-cultured in M9-caseinate medium. The final population sizes of WT PAO1 in 0.25% and 0.1% CAA medium were slightly lower than lasR mutant (Supplementary Fig. 5a), and when the cell densities were equalized, the ATP production of individual WT PAO1 in any CAA concentration was significantly greater than that in lasR mutant (P < 0.01) (Supplementary Fig. 5b). Therefore, these results provided detailed evidence that the emergence of lasR mutants is favored by the population to improve entire fitness using an energy-saving strategy to ease the cooperative burden under nutrient reduction.

**Incorporation of QS-deficient individuals at optimal proportion maximizes population fitness.**

By co-culturing different mixtures of WT PAO1 with lasR mutants, as well as with the randomly selected lasR mutants (lasR2, lasR3, and lasR4) in this study, we found that in the culture with high caseinate level, the whole population size would be shrunken or even collapsed with increasing proportions of lasR mutants (Fig. 6). Notably, when the co-culture was tested with decreasing nutrient levels and the proportion of lasR mutants was about 50%, the whole population size could be maximized. These results suggested that the incorporation of lasR mutants at appropriate proportion can maximize population fitness under nutrient decreased condition.

We then set out to investigate the possibility that an optimal proportion of QS-intact and -deficient individuals might be beneficial for population fitness during infection. We developed acutely and chronically infected mouse models by intranasally instilling different mixtures of WT PAO1 and lasR mutants. For the acute infection model, the survival rate of mice became stable post 5 day’s infection in any case, and the incorporation of lasR mutants caused decreased lethality compared with pure WT PAO1 (Fig. 7a). The residual population that escaped from host lung clearance was maximized when the infection was started with 1:1 mixture of WT PAO1 and lasR mutants (P < 0.05) (Fig. 7b). Compared with the initial instilled proportions, the final proportions of lasR mutants were decreased in all the cases that mice were instilled with different mixtures (Fig. 7c), and this suggested that lasR mutants have a lower fitness than WT PAO1 during acute infection in mouse lungs.

For the chronic infection model, the lung residual CFUs became relatively stable after 7 days except the group infected with pure lasR mutant (Fig. 7d). Such stability indicates the establishment of chronic infection\(^2\). The total population size and composition of *P. aeruginosa* in mouse lung were measured on day 14. Accordingly, the maximal population size was observed in the infection started with 1:1 mixture of WT PAO1 and lasR mutants (P < 0.05) (Fig. 7e). Compared with the initial instilled proportions, the final proportion of lasR mutants was increased in the case that instilled with the mixture containing 25% lasR mutant. This suggested that lasR mutants have a higher fitness than WT PAO1 during chronic infection in mouse lungs. The final proportions of WT PAO1 and lasR mutants were around 50% in the cases that mice were instilled with different mixtures (Fig. 7f). This result combined with the final proportions of lasR mutants in long-term *in vitro* evolution, indicating that the optimal ratio of QS-intact/-deficient individuals for maximizing population fitness might be about 1:1. Consequently, we concluded that the incorporation of lasR mutants into *P. aeruginosa* population units at optimal proportion can maximize population fitness in the colonization of mouse lung, especially during chronic infection.
Discussion
Although the living environments of bacteria are extensive and complex, the flexible genetic characteristic of bacteria may remedy their inferior in cell structure under various environmental pressures. The discovery of QS-coordinated cooperative and competitive behaviors makes the nutrient acquisition and allocation of bacteria.
Figure 7. Optimal mixture of lasR mutants and WT PAO1 maximizes population fitness in mouse lung. (a) Survival rate of mice after acutely infected with gradient mixtures of WT PAO1 and lasR mutant. The residual CFUs (b) and final proportions of WT PAO1 and lasR mutant (c) in acutely infected mouse lungs were enumerated by combined using of LB and M9-adenosin agar on day 5. (d) Residual CFUs in the lungs of chronically infected mouse models. The total residual CFUs (e) and proportions of WT PAO1 and lasR mutant (f) in chronically infected mouse lungs were measured on day 14. Black or white dashed lines indicate the initial proportions of lasR mutants. Mean values ± SEM of one experiment are shown and are representative of three independent experiments. *P < 0.05, One-way ANOVA (Tukey-Kramer post hoc analysis).

more mysterious and attractive28. In this study, we provide experimental evidences regarding the potential molecular mechanism and significance of bacterial QS divergence during evolution.

On the basis of current molecular and growth evidences, we conclude that nutrient reduction can promote de novo emergence of P. aeruginosa lasR mutants under joint effect of RpoS-dominated stringent response and DMMR "switch-off" (Fig. 8). Since LasR plays a central role in the QS cascade, a healthy las QS system under DMMR system supervision can guarantee the intense social cooperation for population fitness under nutrient abundant condition1. However, when the decreased nutrient level cannot support the intense cooperation of the population with high cell density, RpoS will be activated and guiding the entry of population into stationary phase29. The RpoS-dominated stringent response can promote lasR expression to explore potential available nutrient factors by producing more exoproteases (Figs 2d and 3). Therefore, the QS-intact individuals will be caught in a dilemma against continuously decreased nutrient level: insufficient environmental resources can be explored for further highly developed costly cooperation and the imposed pressure to cooperate from RpoS on LasR. On the other hand, increased expression of mutator DinB under RpoS regulation and Hfq-suppressed DMMR system can jointly enhance bacterial genomic plasticity preparing for further adaption in stressed environments29 (Figs 2 and 4). Mutagenesis of lasR gene which is suffering high selection pressure during evolution can perfectly solve this dilemma, as we have noticed that the stable final population size during evolution was expanded with increasing proportions of QS-deficient individuals under nutrient reduced conditions (Fig. 1b).

We did not test the possibility that nutrient reduction may also cause mutagenesis of other genes during evolution but only in lasR, because this study mainly discussed the relationship between nutrient supply and QS system. Previous study using genomic sequencing showed that lasR mutant P. aeruginosa frequently exists in the lungs of CF patients, as well as mutations in other genes with various functions such as antibiotic resistance and virulence13. Additionally, P. aeruginosa from 36% of the CF patients (n = 30) who suffered from pseudomonal infection for years (especially more than 10 years) were hypermutable, whereas this hypermutable feature was not observed in the P. aeruginosa isolated from the lungs of acutely infected non-CF patients (n = 75)12,13. Subsequent studies showed that the frequency of lasR variants was increased in a mutS mutant strain with hypermutable features30,31. These findings also confirmed the association between suppressed function of DMMR system and mutagenesis of lasR gene. The lasR mutants of this study and those identified by Sandoz et al.11 were generated from a single WT colony and did not appear to be hypermutable strains as their mutation frequencies were lower than the criterion for hypermutability (Supplementary Table 5). Hypermutable strains will probably emerge after relatively longer culture periods under conditions of stringent response and DMMR "switch-off".

Comparing with WT PAO1, the productivity of extracellular proteins of lasR mutant is decreased, and this can increase population fitness under limited resources during evolution by reducing the synthetic burden on the cell32. In the conditions with abundant resource supplies, increasing proportions of cheaters will decrease the whole population productivity31. On the contrary, as previously predicted in yeast, food resource will be more efficiently used and optimal mixture of cheaters and cooperators may maximize population benefits19. In this study, we found that the population fitness of P. aeruginosa is dominated by the levels of nutrient supplies and the proportions of lasR mutant cheaters in QS medium (Figs 1b and 6). Specifically, the emergence of cheaters with energy-saving characteristic during evolution is favored by the population to adapt the stationary phase with less resource available. The lasR mutants also possess several superior characteristics such as the utilization...
of particular carbon and nitrogen sources, increased antibiotic resistant ability, as well as avoid cell lysis\textsuperscript{14,34}. All of these evolved phenotypes significantly contribute to the adaption of \textit{P. aeruginosa} in environment of host lung. Because the environment of lung is more complex than \textit{in vitro}, bacterial population may undergo various stresses such as high oxygen content and host immune system\textsuperscript{35}. It is hard to provide a convinced mechanism for the finding that optimal mixture of QS-intact and -deficient individuals will benefit the fitness of whole population \textit{in vivo}, but at least, the energy-saving characteristic of \textit{lasR} mutants may play an important role. Further more comprehensive analyses on the basis of evolved superior characteristics of \textit{lasR} mutants and cell-cell interactions as well as the various host tissue pressures may help to reveal the intrinsic mechanism.

In conclusion, the QS divergence of bacteria is an elaborate evolutionary strategy which can significantly increase the fitness of entire population in tough times. Our current findings enrich the concept of sociomicrobiology and open a new avenue for further investigation of QS-coordinated social divergence, which may also have implications in the development of novel antivirulence strategies for disrupting cell-cell communication in chronic lung infections.

**Materials**

**Bacterial strains.** Wild type \textit{P. aeruginosa} PAO1, QS-deficient \textit{P. aeruginosa} strain PAO1-\textit{ΔlasR}, and the \textit{rpoS} mutant strain PAO1-\textit{ΔrpoS} were gifted from Dr. M. Schuster (Oregon State University, Corvallis, OR, USA) and Dr. S. Lory (Harvard Medical School, Boston, MA, USA)\textsuperscript{36}. PAO1-\textit{ΔdinB} and PAO1-\textit{ΔmutS} were gifted from Dr. D. Wozniak (Ohio State University, Columbus, OH, USA)\textsuperscript{24}. LB broth or designated medium were inoculated with the various strains and incubated at 37 °C.

**In vitro evolution.** Long-term evolution of WT PAO1 was conducted to select \textit{lasR} mutants. Equal amount (1.0 × 10\textsuperscript{7} CFU/ml) of WT PAO1 (which was generated from a single WT colony) was cultured in M9 medium containing different concentrations of caseinate (1%, 0.75%, 0.5%, 0.25%, and 0.1%), and in MOPS-buffered LB broth with gradient dilutions (full-, 1/2-, 1/4-, 1/8-, and 1/16-fold) at 37 °C. All the experiments were performed with shaking (220 rpm) and with or without pre-added QS signal (20 μM 3OC12-HSL). Medium of each culture was refreshed at 24 h interval. For biofilm-related selection, WT PAO1 was inoculated to sterile 12-well polystyrene plates with LB broth (strengths: full, 1/2, 1/4, 1/8, and 1/16) with or without pre-added QS signal. Glass cover slips (20 mm × 20 mm) were used to collect biofilms, and the plates were incubated at 37 °C without shaking. Culture liquids were removed at 24 h interval, and the same size (10 mm × 10 mm) of biofilm was cut from the same portion of glass cover slips for further identification. All the experiments were repeated for 3 times.
To investigate the roles of stringent response and DMMR system in lasR mutation, the rpoS mutant, dinB mutant and mutS mutant strains of WT PAO1 were also conducted for long-term evolution in M9 medium supplied with 0.5% caseinate. Bacteria from M9-caseinate cultures were harvested on days 4, 6, 8, 12, 16, 20, 24, and 30 for further screening of lasR variants. Bacteria from LB broth were harvested on days 7, 14, 21, 28 and 35. The selection and identification of lasR mutants were performed as previously described13. This experiment was also repeated for 3 times independently.

Mutation frequencies of lasR mutants. To investigate whether the identified lasR mutants were hypermutable, their mutation frequencies were determined by exposing to rifampin as previously described by Ciofu et al.36. An isolate was considered hypermutable if the mutation frequency was 20 times higher than that of WT PAO1. All the experiments were repeated for 3 times.

Transcriptome analysis. For global transcriptional analysis, WT PAO1 were cultured in MOPS-buffered LB broth of various strengths (full-, 1/2-, 1/4-, and 1/8-strength). Total RNA was isolated by TRIzol® (Invitrogen) reagents at stationary phase of each culture after 24 h, and the RNA samples from 3 independent cultures were mixed to minimize the deviation of RNA-Seq. The cDNA libraries were constructed and then sequenced using Illumina Hiseq2000 technology. We employed mRNA-Seq for differential expression analysis based on the genome sequence and gene profiles of P. aeruginosa PAO1 (GenBank accession number: AE004091). The software Tophat77 was used to map mRNA sequences to the genome, and subsequently the combination of SOAP2 program38 and Cufflinks39 was used to calculate the expected fragments per kilobase of transcript per million fragments (FPKM) sequenced. Finally, the differentially expressed transcripts were presented and analyzed by edgeR40. The gene expression profiles of P. aeruginosa PAO1 have been deposited at DDBJ/EMBL/GenBank under the accession PRJNA264943.

Quantitative PCR. Total RNA was extracted as described above, and quantitative PCR was performed using the QIAGEN OneStep RT-PCR Kit per the manufacturer’s instructions. The designs of specific primers used in this study (Supplementary Table 6) were based on the consensus of sequences that are deposited in GenBank. Gene expression was calculated by the 2 −ΔΔCT or 2 −ΔΔCT method41 using 16S rRNA as reference.

Competition experiments. LasR mutant and its parent WT PAO1 were co-cultured in 4 ml M9 medium13 containing gradient concentrations of caseinate (1%, 0.75%, 0.5%, 0.25%, and 0%) with or without the addition of 20 μM 3OC12-HSL. The cells of overnight cultured WT PAO1 and lasR mutant, which were started from the inoculation of single colony, were harvested and diluted to optical density (OD600) of 0.01 (corresponding to 2.0 × 10⁷ CFU/ml). The initial lasR mutant to WT PAO1 ratio was 1:99 (1.0 × 10⁷ CFU/ml) and then co-cultured for 36 h. Subsequently, 1 ml of culture liquid was used for plating on LB agar after appropriate dilution. A total of 100 colonies were randomly picked and plated on M9 adenosine agar to enumerate the CFUs of WT PAO1, because lasR mutants are failed to grow in this medium14. Subsequently, a 1:1 mixture of lasR mutant and WT PAO1 was inoculated into gradient M9-caseinate medium and cultured for 36 h to determine the relative fitness of cheaters. The relative fitness (v) was calculated by comparing the initial and final frequencies by using the equation \( v = \frac{x_1(1-x_1)}{x_0(1-x_0)} \), where \( x_0 \) is the initial proportion in the population and \( x_1 \) is their final proportion. The value of relative fitness could indicates the growth rate, for example, \( v = 2 \) means that the growth rate of lasR mutant was twice as fast as WT PAO113. To investigate the effect of lasR mutation on population fitness, gradient mixtures of WT PAO1 and lasR mutants (0, 25%, 50%, 75% and 100%) were inoculated into gradient M9-caseinate medium and cultured for 36 h. All the experiments were repeated for 3 times.

Bioluminescence assay. To test the idea that lasR mutants who escape from QS burden will consume less energy in compare with WT PAO1 under nutrient reduced conditions, equal amount of WT PAO1 and lasR mutants were cultured in M9 medium supplied with different concentrations of CAA as the sole carbon source for 24 h. Production of total ATP during culture was measured by bioluminescence with the ATP Determination Kit (Molecular Probes), in accordance with the manufacturer’s instructions.

Mouse models. Acute lung infection in mice. Gradient ratio of WT PAO1 and PAO1-ΔlasR (pure WT PAO1, 3:1, 1:1, 1:3, and pure lasR mutant) with equal total amounts of CFU 1.0 × 10⁷ were intranasally instilled into C57BL/6J female mice (Indianapolis, IN; weight, 20–25 g, 15 mice per group) that anesthetized by intraperitoneal injection of ketamine (50 mg/ml) and xylazine (5 mg/ml) in 0.9% NaCl. The survival rate was recorded for 7 days. In another independent experiment, mice were killed post 5 days infection and 0.1–0.2 g lung samples from the same part of mouse lungs were aseptically excised and homogenized in 0.9% NaCl. The homogenate was then appropriately diluted and plated onto LB and M9-adenosin agar medium for measurement of the total residual CFU and proportions of WT PAO1 and lasR mutant.

Chronic lung infection in mice. Agar beads containing gradient ratio of WT PAO1 and PAO1-ΔlasR (pure WT PAO1, 3:1, 1:1, 1:3, and pure lasR mutant) were prepared and enumerated as previously described27. The agar beads were diluted into the same inoculum of 1.0–2.0 × 10⁶ CFU in 50μl and then intranasally instilled into anesthetized C57BL/6J female mice (15 mice per group). Mice were killed after 1, 3, 5, 7, 14, and 28 days. The residual CFUs in mouse lungs and proportions of WT PAO1 and lasR mutant were measured as described in acute infection.
Graphpad Prism version 5.0 (San Diego, CA) was used to conduct data analysis and statistical tests. Mean values were compared by using t-test or one-way ANOVA and a subsequent Tukey-Kramer post hoc test using a 95% confidence interval.

All experiments were performed in accordance with the relevant guidelines and regulations, and the entire animal experimental procedures were approved by the Guidance of Institutional Animal Care and Use Committee of Sichuan University (Chengdu, China).

References

1. Darche, S. E., West, S. A., Winzer, K. & Diggle, S. P. Density-dependent fitness benefits in quorum-sensing bacterial populations. Proc. Natl. Acad. Sci. USA 109, 8259–8263 (2012).
2. Fuqua, W. C., Winans, S. C. & Greenberg, E. P. Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. 176, 269–275 (1994).
3. Diggle, S. P., Griffin, A. S., Campbell, G. S. & West, S. A. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450, 411–414 (2007).
4. West, S. A., Griffin, A. S., Gardner, A. & Diggle, S. P. Social evolution theory for microorganisms. Nat. Rev. Microbiol. 4, 597–607 (2006).
5. Ross-Gillespie, A., Gardner, A., West, S. A. & Griffin, A. S. Frequency dependence and cooperation: theory and a test with bacteria. Am. Nat. 170, 331–342 (2007).
6. Dandeker, A. A., Chugani, S. & Greenberg, E. P. Bacterial quorum sensing and metabolic incentives to cooperate. Science 338, 264–266 (2012).
7. Govan, V. & Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol. Mol. Biol. Rev. 60, 539–574 (1996).
8. Latifi, A., Foglio, M., Tanaka, K., Williams, P. & Lazdunski, A. A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhlR (VsrM) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21, 1137–1146 (1996).
9. Schuster, M., Lobohr, C. P., Ogi, T. & Greenberg, E. P. Identification, timing and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J. Bacteriol. 185, 2066–2079 (2003).
10. Dandeker, A. A. & Greenberg, E. P. Plan B for quorum sensing. Nat. Chem. Biol. 9, 292–293 (2013).
11. Sanchez, K. M., Mitzmberg, S. M. & Schuster, M. Social dreaming in Pseudomonas aeruginosa quorum sensing. Proc. Natl. Acad. Sci. USA 104, 13876–13881 (2007).
12. Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. Science 288, 1251–1254 (2000).
13. Smith, E. E. et al. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc. Natl. Acad. Sci. USA 103, 8487–8492 (2006).
14. Heurlier, K. et al. Quorum-sensing-negative (lasR) mutants of Pseudomonas aeruginosa avoid cell lysis and death. J. Bacteriol. 187, 4875–4883 (2005).
15. Wilder, C. N., Diggle, S. P. & Schuster, M. Cooperation and cheating in Pseudomonas aeruginosa: the roles of the las, rhl and pqs quorum-sensing systems. ISME J. 5, 1332–1343 (2011).
16. Hall, A. R. & Colegrave, N. How does resource supply affect evolutionary diversification? Proc. R. Soc. B. 274, 73–78 (2006).
17. Brockhurst, M. A., Buckling, A., Racey, D. & Gardner, A. Resource supply and the evolution of public-goods cooperation in bacteria. BMC Biol. 6, 29 (2008).
18. Eldar, A. Social conflict drives the evolutionary divergence of quorum sensing. Proc. Natl. Acad. Sci. USA 108, 13635–13640 (2011).
19. MacLean, R. C., Fuentes-Hernandez, A., Greig, D., Hurst, L. D. & Gudelj, I. A mixture of "cheaters" and "co-operators" can enable maximal group benefit. PLoS Biol. 8, e1000486 (2010).
20. Lazazera, B. A. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. 3, 177–182 (2000).
21. Wang, M., Schauer, A. L., Dandeker, A. A. & Greenberg, E. P. Quorum sensing and policing of Pseudomonas aeruginosa social cheaters. Proc. Natl. Acad. Sci. USA 112, 2187–2191 (2015).
22. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322 (1999).
23. Kobayashi, H. Airway biofilms: implications for pathogenesis and therapy of respiratory tract infections. Treat. Respir. Med. 4, 241–253 (2005).
24. Layton, J. C. & Foster, P. L. Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in Escherichia coli. Mol. Microbiol. 50, 549–561 (2003).
25. Sanders, L. H. et al. Epistatic roles for Pseudomonas aeruginosa MutS and DinB (DNA Pol IV) in coping with reactive oxygen species-induced DNA damage. PLoS One 6, e18824 (2011).
26. Schuster, M., Hawkins, A. C., Harwood, C. S. & Greenberg, E. P. The Pseudomonas aeruginosa RpoS regulon and its relationship to quorum sensing. Mol Microbiol. 51, 973–985 (2004).
27. Facchini, M., De Fino, L., Riva, C. & Bragonzi, A. Long term chronic Pseudomonas aeruginosa airway infection in mice. J. Vis. Exp. 85, e51091 (2014).
28. Sachs, J. L., Mueller, U. G., Wilcox, T. P. & Bull, J. J. The evolution of cooperation. Q. Rev. Biol. 79, 135–160 (2004).
29. Tsui, H. C., Feng, G. & Winkler, M. E. Negative regulation of mutT and mutH repair gene expression by the Hfq and RpoS global regulators of Escherichia coli K-12. J. Bacteriol. 179, 7476–7487 (1997).
30. Harrison, F. & Buckling, A. Hypermutability impedes cooperation in pathogenic bacteria. Curr. Biol. 15, 1968–1971 (2005).
31. Lujan, A. M., Moyano, A. J., Segura, I., Argañara, C. E. & Smania, A. M. Quorum-sensing-deficient (lasR) mutants emerge at high frequency from a Pseudomonas aeruginosa mutS strain. Microbiology 153, 225–237 (2007).
32. Smith, D. R. & Chapman, M. R. Economical evolution: microbes reduce the synthetic cost of extracellular proteins. mBio 1, e00131–10 (2010).
33. Popat, R. et al. Quorum-sensing and cheating in bacterial biofilms. Proc. R. Soc. B. 279, 4765–4771 (2012).
34. D’Argenio, D. A. et al. Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. Mol. Microbiol. 64, 512–533 (2007).
35. Yonker, L. M., Cigana, C., Hurley, B. P. & Bragonzi, A. Host-pathogen interplay in the respiratory environment of cystic fibrosis. J. Cyst. Fibros. 14, 431–439 (2015).
36. Ciofu, O., Riis, B., Pressler, T., Poulsen, H. E. & Haiby, N. Occurrence of hypermutable Pseudomonas aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrob. Agents Chemother. 49, 2276–2282 (2005).
37. Trappell, C., Pachtet, L. & Saleberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111 (2009).
38. Li, R. et al. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25, 1966–1967 (2009).
39. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628 (2008).
40. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2010).
41. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45 (2001).

Acknowledgements
This work was supported by the Science and Technology Support Plan of Sichuan Province (2014NZ0107 to B.Y.), the National Basic Research Program of China (973 Project 2012CB722207 to X.Zhang), and the China Scholarship Council (grant 201206240130 to K. Z.).

Author Contributions
K.Z., B.Y. and X.Z. designed the work; K.Z. performed all the experiments and wrote the manuscript; X.Z. provided animals and coordinated animal experiments; W.L. performed the bioinformatics analyses; K.Z., B.Y. and X.Z. coordinated manuscript writing.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhao, K. et al. Nutrient reduction induced stringent responses promote bacterial quorum-sensing divergence for population fitness. Sci. Rep. 6, 34925; doi: 10.1038/srep34925 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2016