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ORIGINAL ARTICLE

Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating

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Quorum sensing (QS) coordinates the expression of virulence factors and allows bacteria to counteract the immune response, partly by increasing their tolerance to the oxidative stress generated by immune cells. Despite the recognized role of QS in enhancing the oxidative stress response, the consequences of this relationship for the bacterial ecology remain unexplored. Here we demonstrate that QS increases resistance also to osmotic, thermal and heavy metal stress. Furthermore a QS-deficient *lasR rhlR* mutant is unable to exert a robust response against H_2O_2 as it has less induction of catalase and NADPH-producing dehydrogenases. Phenotypic microarrays revealed that the mutant is very sensitive to several toxic compounds. As the anti-oxidative enzymes are private goods not shared by the population, only the individuals that produce them benefit from their action. Based on this premise, we show that in mixed populations of wild-type and the *mexR* mutant (resistant to the QS inhibitor furanone C-30), treatment with C-30 and H_2O_2 increases the proportion of *mexR* mutants; hence, oxidative stress selects resistance to QS compounds. In addition, oxidative stress alone strongly selects for strains with active QS systems that are able to exert a robust anti oxidative response and thereby decreases the proportion of QS cheaters in cultures that are otherwise prone to invasion by cheats. As in natural environments stress is omnipresent, it is likely that this QS enhancement of stress tolerance allows cells to counteract QS inhibition and invasions by social cheaters, therefore having a broad impact in bacterial ecology.
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Introduction

Quorum sensing (QS) is cell communication used by bacteria to coordinate their behavior and the expression of several phenotypes once a population size threshold is reached. For example, it controls the expression of multiple virulence factors and associated behaviors such as swarming and biofilm formation in several bacterial pathogens, including *Pseudomonas aeruginosa* (Dunny and Leonard, 1997; Jayaraman and Wood, 2008; Antunes *et al.*, 2010). In addition, QS protects bacterial pathogens against the immune system. With *P. aeruginosa*, its main autoinducer signal, *N*-(3-oxododecanoyl)-homoserine lactone, inhibits lymphocyte proliferation and the secretion of several cytokines by macrophages and T-cells (Telford *et al.*, 1998). QS also allows *P. aeruginosa* biofilms to inhibit phagocytosis and oxygen radical bursts by neutrophils (Bjarnsholt *et al.*, 2005). Furthermore, the activities of QS-controlled virulence factors such as elastase, alkaline protease and rhamnolipids interfere with the activation of phagocytosis and cell signaling (Leid *et al.*, 2005; Kuang *et al.*, 2011; Dössel *et al.*, 2012; Laarman *et al.*, 2013). The relationship between QS and resistance of biofilms to several antibiotics like tobramycin is also well documented (Davies *et al.*, 1998; Hentzer *et al.*, 2003).

Beyond the link of QS to antibiotic resistance, for *P. aeruginosa*, a direct link between QS and stress tolerance is the fact that QS-deficient mutants (*lasI*, *rhlI* and *lasI rhlI*) have defective expression of *katA* and *sodA* and concomitantly less catalase (CAT) and superoxide dismutase (SOD) activities, being therefore more sensitive to oxidative stress than

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the parental strain (Hassett *et al.*, 1999). Also for *P. aeruginosa*, the H_2O_2-responsive transactivator OxyR binds the promoters and may influence the expression of the QS transcriptional regulators *rsaL* and *mvfR* (*pqsR*) (Wei *et al.*, 2012), and nutritional stress by phosphate starvation and QS are linked (Lee *et al.*, 2013).

Although some molecular details about the role of QS autoinducers and virulence factors in increasing tolerance against the immune system and antimicrobials are well studied, the concerted response of all the QS determinants against the host attack or harsh environmental conditions is not yet well understood. Indeed, our present knowledge suggests that a significant subset of QS-regulated genes is related to stress tolerance (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). Noteworthy, most of the proteins encoded by these genes have not yet well-defined functions. The role of QS in enhancing the stress tolerance against abiotic factors in other bacteria such as *Vibrio* species and *Burkholderia pseudomallei* has also been demonstrated (McDougald *et al.*, 2003; Lumjiaktase *et al.*, 2006; Joelsson *et al.*, 2007; Defoirdt *et al.*, 2010).

Moreover, the role of QS in the survival of bacteria during biotic stress has recently been investigated. For example, functional QS systems confer *P. aeruginosa* with an elevated resistance to predation by protists, apparently by promoting the formation of predation-resistant biofilms in contrast to those produced by *lasI* and *lasR* HSL QS-deficient mutants (Friman *et al.*, 2013). Also, in iron-limited media, the production of the siderophore pyoverdine by *P. aeruginosa* can be exploited by mutants as long as they produce less siderophore than the cooperator cells that are being exploited (Ghoul *et al.*, 2014).

In general, QS-regulated phenotypes are energetically costly, and if they are performed by only a few cells, their expression can be detrimental rather than beneficial for the individuals and the population (Diggle *et al.*, 2007; Rutherford and Bassler, 2012). In principle, the production of extracellular factors regulated by QS, like siderophores and proteases, can benefit all the members of the population regardless if they cooperate in their production or not and are therefore considered public goods (Diggle *et al.*, 2007). In addition, QS also controls the expression of a few metabolic processes involving the production of intracellular enzymes not shared by the population, such as for adenosine catabolism in *P. aeruginosa* (Heurlier *et al.*, 2005); these enzymes are therefore private goods. Recently, the utilization of adenosine as a sole carbon source (that is, a private good) allowed us to isolate the first mutants resistant to a QS inhibitor, the brominated furanone C-30; these mutants have the gene *mexR* disrupted that encodes an efflux repressor, so they are able to efflux C-30 by the MexAB-OmpR multidrug efflux pump (Maeda *et al.*, 2012). Also, recently it was demonstrated that the addition of adenosine to *P. aeruginosa* cultures with caseinate as the sole carbon source reduces the frequency of social cheaters that are *lasR* mutants that do not cooperate with the production of the proteases necessary for the cleavage of casein but that consume the amino acids and peptides released by the protease produced by the cooperative individuals (Dandekar *et al.*, 2013).

In this work, in addition to showing that QS systems are related to protecting the cells against a wide-range of environmental stresses (for example, to osmotic, thermal and heavy metal stress); we find three interesting features related to bacterial ecology. First, we show that as the *P. aeruginosa* anti oxidative stress response is enhanced by QS and involves the production of private goods (that is, intracellular enzymes, such as CAT and NADPH-producing dehydrogenases), the individuals that produce these private goods are able to tolerate better the addition of H_2O_2; hence, oxidative stress selects for cells that have an active QS system. Second, this oxidative stress also selects for more *mexR* C-30-resistant mutants under quorum quenching treatment; hence, oxidative stress selects for cells that are resistant to QS inhibitors. Third, oxidative stress decreases the proportion of protease-putative QS cheaters in cultures with caseinate as the sole protein source. Hence, the role of QS in regulating stress tolerance has potentially important consequences for the bacterial physiology and ecology.

Materials and methods

All strains used are listed in Table 1. Cells were cultured at 37 °C with gentamicin at 15 μgml^{-1} or tetracycline at 75 μg/ml^{-1} for the selection of mutants.

Stress tolerance experiments

The assays were adapted from (Zhang *et al.*, 2007). Briefly, strains were cultured in Luria Broth (LB) from overnight precultures, until the cultures reached a turbidity at optical density (OD) 600 nm of ~1.5 and then aliquots of 1 ml were taken and stressed as follows: (a) heat shock: 10 min at 65 °C, (b) oxidative stress: H_2O_2 was added at a final concentration of 200 mM, and the cells were exposed for 30 min at 37 °C without shaking, (c) heavy metal exposure: $CdCl_2$ was added at a final concentration of 16 mM, and the cells were exposed for 20 min at 37 °C without shaking, and (d) osmotic shock: cells were centrifuged at 13 000 r.p.m. for 1.5 minutes, resuspended in 4 M NaCl, and incubated for 30 min at 37 °C without shaking. For the furanone C-30 experiments, wild-type or *mexR* cultures were grown until mid-exponential phase (OD 600 nm of ~0.5) and then C-30 was added at 50 μM (as a negative control, ethanol, the C-30

Table 1 Bacterial strains used in this study

Bacterial strains	*Genotype/relevant characteristics*	*Source*
PA14	*Pseudomonas aeruginosa* wild-type	(Liberati *et al*., 2006) and (Park *et al*., 2005)
lasI	PA14 *pqsA*Ω Mar2xT7, Gm^R	(Liberati *et al*., 2006)
rhlI	PA14 *pqsA*Ω Mar2xT7, Gm^R	(Liberati *et al*., 2006)
pqsA	PA14 *pqsA*Ω Mar2xT7, Gm^R	(Liberati *et al*., 2006)
lasR	PA14 Δ*lasR*; Rif^R	(Park *et al*., 2005)
rhlR	PA14 *rhlR*Ω Mar2xT7, Gm^R	(Liberati *et al*., 2006)
mvrF	PA14 *mvrF*Ω Mar2xT7, Gm^R	(Liberati *et al*., 2006)
lasR rhlR	PA14 Δ*lasR*Δ*rhlR*; Rif^R	(Park *et al*., 2005)
mexR	PA14 *mexR*Ω miniTn5*luxAB*-Tet, Tc^R	(Maeda *et al*., 2012)

Gm^R, Rif^R and Tc^R are gentamicin, rifampicin and tetracycline resistance, respectively.

vehicle diluent, was added for comparison). Cells were then grown until the cultures reached turbidity at OD 600 nm of ~1.5 and stressed. After exposure, serial dilutions and viable counts were done to determine viability by colony-forming units. Unstressed cultures at an OD 600 nm of ~1.5 were used to determine the initial colony-forming units before the stress. All experiments were done at least in triplicate.

Competition experiments between wild-type strain and mexR

The wild-type (sensitive to C-30) and *mexR* (C-30 resistant) strains were cultured in LB to a turbidity of 600 nm of ~0.5 and then mixed at proportions 1:1 or 1:10 *mexR*: wild-type in LB medium. C-30 at 50 μM (or the vehicle ethanol as a negative control) was added, and then cells were grown to turbidity at OD 600 nm of ~1.5 (at this point, viable counts were done to determine the initial strain proportions). H_2O_2 (160 mM) was added for 30 min of exposure, and viable counts were done in LB. For the quantification of the *mexR* proportion, colonies obtained in LB were patched on LB with 20 $\mu g ml^{-1}$ tetracycline (as only *mexR* mutants can grow in the presence of this antibiotic).

Also a similar experiment but including five serial culture passes was performed. For this experiment, the wild-type strain and *mexR* mutant were mixed at an initial ratio of 1:4, and the following treatments were administrated: (1) control (ethanol, the C-30 vehicle), (2) ethanol + 80 mM H_2O_2, (3) C-30 at 50 μM, and (4) C-30 at 50 μM + 80 mM H_2O_2). Cell passes were made every 24 h, and the cultures were set to an initial turbidity of OD 600 nm of ~0.05. Ethanol or C-30 was administrated when cells reached a turbidity of OD 600 of ~0. 5 and H_2O_2 when the turbidity was ~1.5. The *mexR* proportion at the beginning of the experiment and before each new culture pass was assessed by plating colonies in tetracycline as described previously. The Malthusian rates (relative fitness) for both strains were calculated by the natural logarithm of the ratio of final (after the H_2O_2 challenge) and initial cell densities of the strains (Lenski *et al*., 1991). Experiments were done in quadruplicate.

Effect of H_2O_2 stress in QS^- social cheaters dissemination

The PA14 wild-type strain was cultured in 5 ml of M9 medium at pH 7.0 with 0.5% casein as the sole carbon source. Cells (200 μl) were transferred to a new medium every 24 h, and the proportion of social cheaters was determined by plating colonies on LB and transferring a fraction of the colonies to plates supplemented with 3% skim milk and minimal M9 plates with adenosine as the sole carbon source. Colonies unable to produce a proteolysis halo on the LB milk plates and unable to grow on adenosine were considered putative QS-negative social cheaters. In order to verify whether those colonies were QS negative, nine were chosen randomly and their elastolytic, alkaline protease, pyocyanin and pyoverdine activities were determined (García-Contreras *et al*., 2013b). The pH of the medium was determined after each pass, and it remained around 7.0. Hence the enrichment in cheater proportions was not due to an increase in pH of the medium, which allows higher survival of *lasR* mutants compared with parental wild-type strain (Heurlier *et al*., 2005). To test the effect of exposure to severe oxidative stress in the cheater survival, after nine consecutive culture passes, cells were stressed with H_2O_2 (160 mM) for 30 min and the tenth consecutive culture pass was done using 0.5 ml of the stressed cells as inocula. The cheater proportion was determined for the tenth pass and the following 10 passes. Similarly, previously stressed cultures were exposed to additional H_2O_2 at pass 14. In order to test the effect of moderate oxidative stress; H_2O_2 (40 mM or 60 mM) was added immediately after the inoculation from passes 3 to 10. Experiments were done in triplicate.

Statistical analysis

All experiments were done at least in triplicate; values are expressed as mean ± s.e.m. The normal distribution of the data was determined by a Kolmogorov–Smirnov test. Statistically significant differences between the mutants and the wild-type strain and between the control and treatments for the data shown in Figures 1–3 and for the data shown in Tables 2A, B and 3 were determined by a

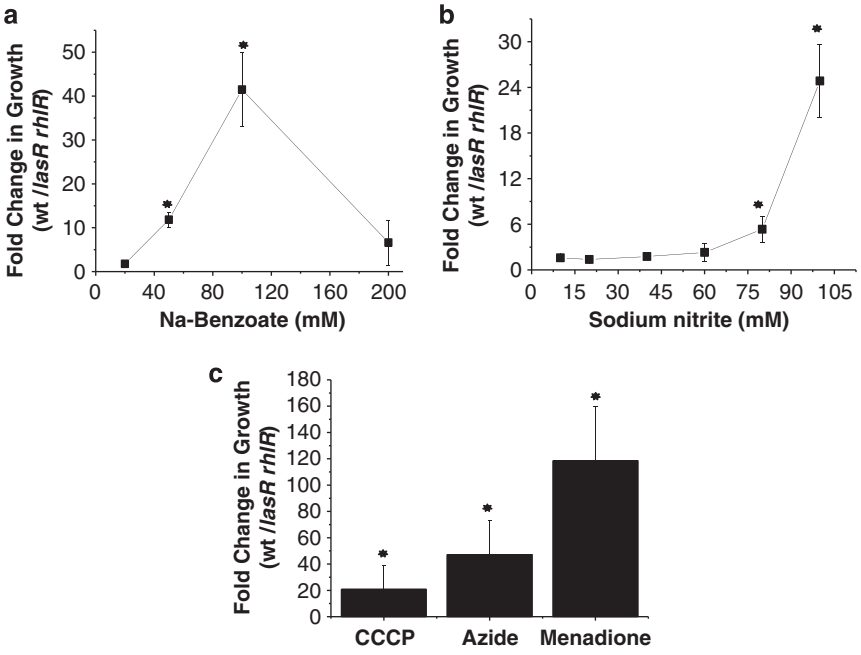

Figure 1 Phenotypic microarrays show the *lasR rhlR* mutant is more sensitive to toxic compounds than the wild-type strain. Effects of (**a**) benzoate, (**b**) nitrite, (**c**) CCCP, azide and menadione on growth. Fold change in growth (wild-type/*lasR rhlR* mutant) is shown. The experiments were done according to the manufacturer's instructions (Biolog), using the PM9 and PM15B plates. Significant differences ($P<0.05$, Student's two-tailed test comparing wild-type and *lasR rhlR* mutant growths) are shown by asterisks.

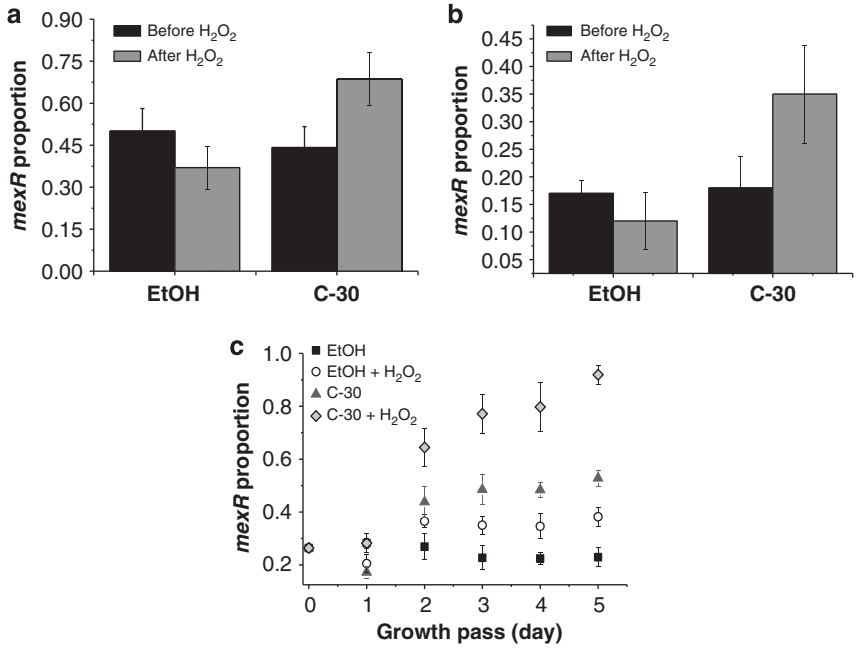

Figure 2 Challenge with H_2O_2 increases the proportion of *mexR* cells after a treatment with the quorum quencher furanone C-30. Initially, wild-type (QS^- in the presence of C-30) and *mexR* (QS^+ in the presence of C-30) cultures were mixed at (**a**) 1:1 or (**b**) 1:10 *mexR* proportions. Cells were then co-cultured in the presence of either C-30 (50 μM) or ethanol, the furanone diluent. When cultures reached the late stationary phase, H_2O_2 was added. Viable counts in LB with tetracycline were done to estimate the *mexR* proportion before and after H_2O_2 addition. For (**c**), the *mexR* and wild-type strains were mixed at an initial proportion of 1:4, and five consecutive culture passes were done (for further experimental details, see Material and methods). The average and s.e.m. of four independent experiments are shown. For panels (**a** and **b**), significant differences ($P<0.05$, Student's two-tailed between the initial and final proportions of *mexR*) were found for the treatments, including C-30 and H_2O_2. For panel (**c**), all differences between control (ethanol) and the three treatments (C-30, H_2O_2 or C-30 + H_2O_2) were significant ($P<0.05$, Student's two-tailed test between the initial and final proportions of *mexR*) from passes 3, 4 and 5.

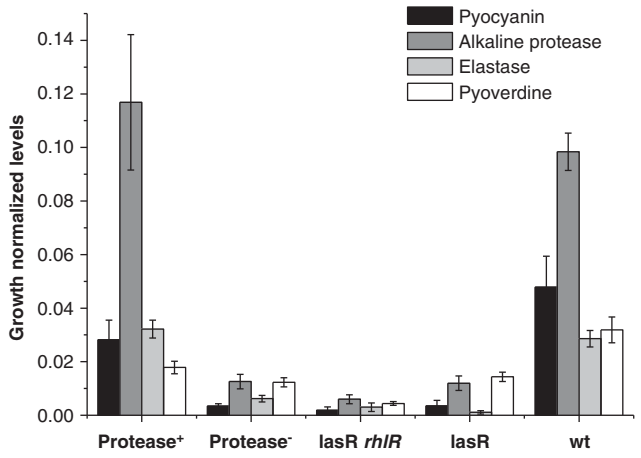

Figure 3 Quorum sensing-controlled expression of virulence factors is much lower in protease$^-$ clones (putative social cheaters) than in protease$^+$ clones (putative cooperators). The activity of pyocyanin, alkaline protease, elastase and pyoverdine was determined in nine independent protease$^+$ clones (putative cooperators) and nine independent protease$^-$ clones (putative cheaters). Three independent cultures of wild-type, *lasR* mutant and *lasR rhlR* mutant are presented as controls. Average and s.e.m. are shown. All phenotypes showed significant differences ($P<0.05$, Student's two-tailed test) between wild-type and cheaters, *lasR* and *lasR rhlR* mutants. Except for pyoverdine, differences were not significant between the wild-type strain and the protease$^+$ clones.

Student's two-tailed test and considered significant if $P<0.05$. For Figure 4, data was fitted to a logistic regression model or to a logistic plus inhibition model using the OriginPro 8.5.1 software (OriginLab Corporation, Northampton, MA, USA), and statistical significance was assessed by an analysis of variance (ANOVA) test and a *post hoc* Tukey's Honestly Significant Differences test; differences were considered significant if $P<0.05$. All statistical analyses were done with the IBM-SPSS 20v software (IBM, Armonk, NY, USA).

Results and Discussion

Disruption of QS systems severely decrease stress tolerance

To further expand the evidence of the role of QS systems in stress tolerance, the survival upon stress of PA14 isogenic QS autoinducer receptor mutants, *lasR*, *rhlR* and *mvrF*, and a double *lasR rhlR* mutant, was tested. The selected stressors were heat, oxidative stress, heavy metals and hyperosmolarity. Single *lasR* or *rhlR* mutants survival after stress was ~3-fold lower than wild-type survival for all four stresses, while the double mutant was 4.8- to 11.2-fold more sensitive. In contrast, *mvfR* mutant survival was not different from the wild type (Table 2A), indicating that the *P. aeruginosa* QS system dependant on quinolone signals is either not related to stress responses or otherwise not active under the tested experimental conditions. This is consistent with the fact that PQS is produced maximally at late stationary phase (McKnight *et al.*, 2000), while our experiments were done at the end of exponential phase/early stationary phase. Therefore, in addition to oxidative stress, the RhlR and LasR QS systems are related to heat, heavy metal and salt stress.

Table 2A Ratio of the viability of the wild-type strain vs QS-deficient mutants after exposure to stress

Strain	*Heat*	*H_2O_2*	*$CdCl_2$*	*NaCl*
lasR	3 ± 1.5	3.1 ± 1.3*	3.2 ± 0.9*	3 ± 0.6*
rhlR	4.4 ± 1.2*	2.8 ± 0.05*	2.6 ± 1.0	2.7 ± 0.8*
mvfR	1.2 ± 0.1	1.1 ± 0.02	1.2 ± 0.2	1.1 ± 0.05
lasR rhlR	6.7 ± 0.9*	11.2 ± 5.0*	6.5 ± 0.92*	4.8 ± 2.2*

Abbreviation: QS, quorum sensing.

Table 2B Ratio of the viability of PA14 wild-type and *mexR* mutant without C-30 relative to with C-30 treatment after exposure to stress

Strain	*Heat*	*H_2O_2*	*$CdCl_2$*	*NaCl*
PA14 + C30	6.7 ± 1.2*	5.4 ± 1.4*	8.2 ± 1.9*	3.0 ± 0.6*
mexR + C30	1.1 ± 0.5	1.1 ± 0.1	1.3 ± 0.4	1.5 ± 0.6

Mean values and s.e.m. of four independent experiments are shown. Significant differences ($P<0.05$, Student's two-tailed test) comparing wild-type and *lasR rhlR* mutant growths for Table 2A and wild-type or *mexR* growths without and with C-30 treatments for Table 2B) are shown by asterisks.

To corroborate that the RhlR and LasR QS systems are related to stress, the effect of furanone C-30, a canonical QS inhibitor (Ren *et al.*, 2001; Hentzer *et al.*, 2003), was tested in the stress resistance of the PA14 strain by adding 50 μM of C-30 to the cultures. As expected based on results from the cells with QS mutations, wild-type cells (QS$^-$ in the presence of C-30) became 3- to 8.2-fold more sensitive toward the four tested stress conditions due to suppression of QS by C-30. Moreover, the isogenic *mexR* mutant, which is able to efflux C-30 and hence is resistant against this quorum quencher (QS$^+$ in the presence of C-30) (Maeda *et al.*, 2012), only slightly increases its sensitivity to stress with C-30 treatment (Table 2B).

To further explore the effects of HSL-QS systems' loss in the tolerance against stress, phenotypic microarrays comparing the PA14 wild-type strain and the *lasR rhlR* mutant were conducted using the Biolog plates (Biolog Inc, Hayward, CA, USA) PM9, P11C and PM15B, which include several different stressors (osmolytes, antibiotics, chelators, metabolism inhibitors and so on). For PM9, after 24 h of incubation, there was up to 29-fold greater growth for the wild-type strain. Remarkably, the wild-type strain was much better at resisting stress produced by sodium nitrite and benzoate than the QS-deficient mutant (Figures 1a and b). For plate PM15B, a similar trend was observed, as the

Table 3 Activities of enzymes related to stress response

Enzyme	*Late exponential phase*		*Stationary phase*	
	Wild type	*lasR rhlR*	*Wild type*	*lasR rhlR*
Catalase	13 600 ± 8000	13 683 ± 791	14 903 ± 1947	7452 ± 1093[a]
+ C-30	6028 ± 1398	ND	ND	ND
H_2O_2, 20 mM	80 252 ± 34 060[b]	20 394 ± 2720[b]	ND	ND
H_2O_2, 80 mM	41 939 ± 1274[b]	14 642 ± 2270[a]	ND	ND
H_2O_2, 160 mM	39 220 ± 13 475[c]	6443 ± 1267[a,b]	ND	ND
H_2O_2, 160 mM + C-30	3921 ± 908[c]	ND	ND	ND
SOD	17.5 ± 4.6	13.2 ± 2.8	16.6 ± 11	36.5 ± 21
+ C-30	10.5 ± 5.4	ND	ND	ND
H_2O_2, 20 mM	5.6 ± 4.1	4.3 ± 4.2	ND	ND
H_2O_2, 80 mM	0.86 ± 0.86	0 ± 0[b]	ND	ND
H_2O_2, 160 mM	0.28 ± 0.28[b]	0 ± 0[b]	ND	ND
H_2O_2, 160 mM + C-30	0 ± 0	ND	ND	ND
ME	39 ± 3	23 ± 10[a]	20 ± 4	10 ± 1.6[a]
H_2O_2 20 mM	30 ± 2[b]	11 ± 2[a]	ND	ND
H_2O_2 80 mM	43 ± 3	16 ± 4[a]	ND	ND
H_2O_2 160 mM	61 ± 11	10 ± 2[a]	ND	ND
G6PDH	16 ± 6	15 ± 3	2.6 ± 0.3	2.4 ± 0.6
H_2O_2, 20 mM	20 ± 3	22 ± 2	ND	ND
H_2O_2, 80 mM	17 ± 3	13 ± 3	ND	ND
H_2O_2, 160 mM	13 ± 4	11 ± 4	ND	ND
IDH	470 ± 30	443 ± 202	495 ± 49	442 ± 50
+ C-30	556 ± 26[c]	ND	ND	ND
H_2O_2, 20 mM	641 ± 6[b]	588 ± 29[a]	ND	ND
H_2O_2, 80 mM	1026 ± 217[b]	560 ± 1[a]	ND	ND
H_2O_2, 160 mM	904 ± 181[b]	378 ± 54[a]	ND	ND
H_2O_2, 160 mM + C-30	394 ± 31[c]	ND	ND	ND

Abbreviations: G6PDH, glucose-6 phosphate dehydrogenase; IDH , isocitrate dehydrogenase; ME, malic enzyme; ND, not determined; SOD, superoxide dismutase.
Activities in nmol substrate per min mg protein, except for SOD (SOD units per mg protein) mean values of three independent experiments and s.e.m. are shown.
[a]Mean significant difference $P<0.05$ two-tailed Student's *t*-test vs same treatment in wild-type.
[b]Mean significant difference $P<0.05$ two-tailed Student's *t*-test vs control without H_2O_2.
[c]Mean significant difference $P<0.05$ two-tailed Student's *t*-test vs treatment without C-30.

wild-type strain grew up to 120-fold greater than the *lasR rhlR* mutant in the presence of menadione (Figure 1c). For the P11C plate (antibiotics), there was no appreciable growth of any strain after 10 days of incubation.

The compounds that showed the higher growth differences like benzoate and carbonyl cyanide-m-chlorophenylhydrazone (CCCP), are uncouplers of the oxidative phosphorylation, and at least for CCCP its uncoupling activity has been demonstrated for *P. aeruginosa* (Ikonomidis *et al.*, 2008). Azide is an inhibitor of complex IV of the respiratory chain, the enzyme cytochrome oxidase, and menadione is a compound that produces oxidative stress (Smirnova *et al.*, 2000; Criddle *et al.*, 2006). The effect of all these compounds could converge in an enhanced generation of reactive oxygen species (ROS) or reactive nitrogen species in the case of nitrite, as azide can oxidize NADH in submitochondrial particles to greatly increase the production of H_2O_2 (Chen *et al.*, 2003) and is an inhibitor of CATs (Thurman and Chance, 1969). Incubation with uncouplers like CCCP increases the ROS generation (Izeradjene *et al.*, 2005) and nitrite can be reduced to nitric oxide, which reacts with oxygen to produce reactive nitrogen species (Takahama *et al.*, 2005). Together, these results serve to show clearly that there is a wide range of stress for which it is beneficial for the cell to have active QS systems.

QS enhances the oxidative stress response through the production of antioxidant enzymes

The diminished expression of CAT and SOD expected in the *lasR rhlR* mutants (Hassett *et al.*, 1999) could be related to its high sensitivity to ROS stress. Consistent with this hypothesis, the basal CAT activity of the wild-type strain at stationary phase (OD 600 nm ~ 3.0) was twofold higher than the activity of the *lasR rhlR* mutant. In contrast, basal SOD activity was twofold higher in the mutant. NADPH provides the reductive power for the detoxification of ROS by glutathione peroxidase/reductase (Perry *et al.*, 1991) and protects CAT from inactivation by its substrate, H_2O_2 (Kirkman *et al.*, 1999). Therefore, the activity of the NADPH-

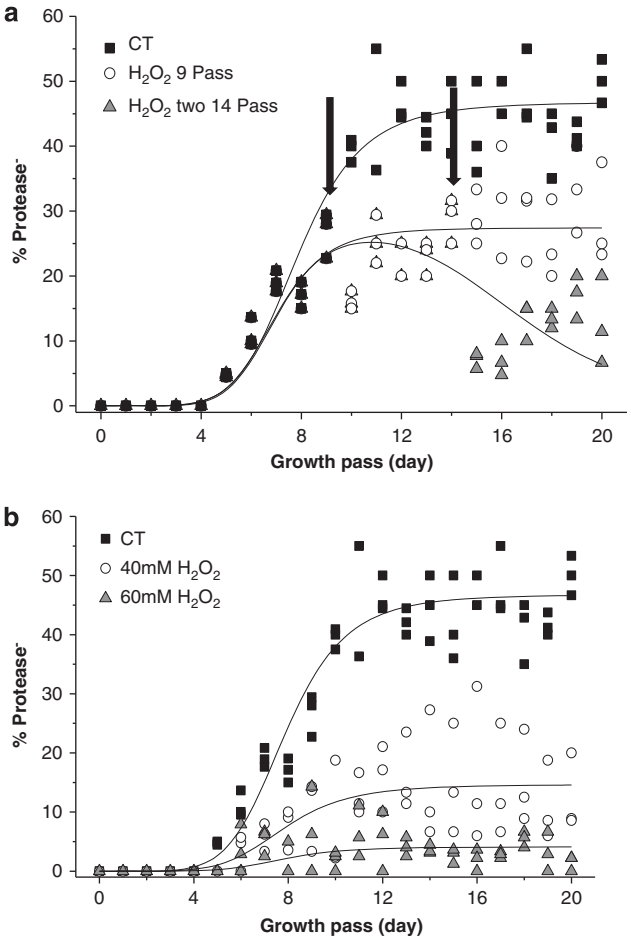

Figure 4 The addition of H_2O_2 decreased the proportion of putative social cheaters in cultures with casein as sole carbon source. (**a**) Effect of high concentration H_2O_2 pulses, and arrows indicate the addition of 160 mM H_2O_2. Data from control and one pulse of H_2O_2 were fitted to a logistic regression model, and data from two pulses of H_2O_2 were fitted to a logistic plus inhibition model. ANOVA analysis shows significant differences between untreated and H_2O_2-treated cultures ($P<0.05$, $F=17.7$ ANOVA test) at 10, 15 and 20 daily growth passes. (**b**) Effect of moderate and continuous addition of H_2O_2; it was administrated at 40 mM and 60 mM from pass 3 to pass 20. Individual data of three independent cultures are shown. ANOVA analysis shows significant differences between untreated and H_2O_2-treated cultures ($P<0.05$, $F=34$ ANOVA test) at 10, 15 and 20 daily growth passes. All logistic regressions were made using the OriginPro 8.5.1 software.

producing enzymes for isocitrate, malate and glucose 6-phosphate was determined; with the result that malate dehydrogenase (malic enzyme (ME)) activity was also twofold higher for the wild-type strain. In contrast, the activities of isocitrate dehydrogenase (IDH) and glucose-6 phosphate dehydrogenase (G6PDH) were not different between the wild-type and mutant, and as expected, G6PDH was low for both strains, as in LB medium there are no substrates that induce its activity (glucose, glycerol and so on), and activity is low in stationary phase (Ma *et al.*, 1998). Note that in agreement, the activity of G6PDH for both strains was fourfold higher in the late exponential phase than in the stationary (Table 3). Moreover, when the cells were stressed with H_2O_2 (20, 80 or 160 mM) at the end of the exponential growth phase, for the wild-type strain the activity of CAT increased sixfold with the addition of 20 mM of H_2O_2 and remained threefold higher than without the stressor at 80 and 160 mM of H_2O_2. In contrast, the *lasR rhlR* mutant CAT increased only 1.5-fold with 20 mM of H_2O_2, had no change with 80 mM and decreased 50% at 160 mM. Similarly, wild-type ME increased 1.6-fold with 160 mM of H_2O_2, while for the mutant it decreased −1.79-fold, and IDH was induced ~2-fold with 80 mM and 160 mM of H_2O_2 for the wild-type strain, while in the mutant it only was induced 1.35-fold with 20 mM and 80 mM of H_2O_2 and decreased −1.18-fold with 160 mM. In contrast, the basal activity and the changes in G6PDH activity upon H_2O_2 addition were similar in both strains. Finally, SOD activity decreased in both strains after the addition of H_2O_2, but the decrease was more severe in the mutant as activity completely disappeared with 80 mM and 160 mM of H_2O_2, while for wild type 50% and 16% remained, respectively (Table 3). The effect of C-30 on the activities of CAT and IDH (the NADPH-producer enzyme with the higher activity and induction with H_2O_2) were also evaluated, with the result that the addition of C-30 to the wild-type strain inhibited 50% the CAT activity in the absence of H_2O_2 and 90% in the presence of 160 mM of H_2O_2. For IDH in the absence of H_2O_2, the addition of C-30 slightly increased its activity (1.18-fold), but in the presence of H_2O_2 activity was inhibited 57%. Similarly, C-30 decreased SOD activity 40% in the absence of H_2O_2 and 100% in its presence (Table 3). Taken together, our results demonstrate that the enzymatic response against ROS in the wild-type strain is stronger than in the QS-deficient mutant and the addition of the quorum quencher C-30 inhibits the antioxidant defense.

In order to further explore the influence of QS integrity on the development of a robust oxidative stress response, DNA microarrays of the *lasR rhlR* mutant were done, first in basal conditions during the late exponential phase (growing aerobically at 37 °C to turbidity at OD 600 nm of ~1.5). The results show that a total of 51 genes were induced at least 3-fold in the mutant while 76 were repressed, and as expected among the repressed genes *lasR* and *rhlR* (of which at least 60% of their sequence was deleted in the mutant (Park *et al.*, 2005)) were −11- and −52-fold repressed, respectively, while *lasI* and *rhlI* were −17- and −6-fold repressed. In addition, the QS regulator *rsaL* was repressed −32-fold and six PQS biosynthesis genes *pqsABCDEH* were repressed between −6- and −30-fold, corroborating the QS deficiency of the mutant. Also, in agreement with previous findings, several genes related to virulence factors were repressed. In agreement with the importance of QS for the expression of several stress-related genes, 11 genes previously identified as being induced by QS and that have known or putative stress related functions were also repressed

at least twofold in the mutant in basal conditions (Supplementary Table S1).

In addition to determining the global gene expression in basal conditions, the transcriptome was determined after exposing late exponential phase cells to 80 mM H_2O_2 for 30 min. In this condition, 478 genes were induced, and 66 were repressed at least threefold in the mutant (Supplementary Table S2). Interestingly, of the 11 stress-related QS-controlled genes found repressed in the mutant at basal conditions, 10 remained repressed after the challenge with H_2O_2 (Supplementary Table S2), which may be linked to the *lasR rhlR* mutant's high sensitivity towards H_2O_2-promoted damage. Genes encoding the enzymes studied: CAT (*katA*, *katB*), SOD (*sodB*), ME (*phaC1*, PA3471), G6PDH (*zwf*), and IDH (*idh*, *icd*), were not expressed differentially in the wild-type and in the mutant neither in the basal conditions nor after the H_2O_2 challenge. This does not correlate with the induction of CAT and IDH activities in the wild-type strain by 80 mM H_2O_2; nevertheless, *katA*, *katB*, *idh* and *icd* were found not to be directly induced by QS in three independent microarray studies (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). In contrast, *sodM*, encoding manganese-dependant SOD, was induced in both strains but to a higher extent in the *lasR rhlR* mutant (fivefold higher induction than in the wild type); however, the activity of SOD for the mutant with 80 mM H_2O_2 was completely abolished, regardless of the induction of *sodM* (Table 3). In addition, both strains induced *katA* and *katB* genes, 6.5- and 2.8-fold in the wild type and 7.4- and 4.3-fold in the mutant, respectively (Supplementary Table S1). In summary, our results show that the production of several private goods, such as the enzymes used to contend with oxidative stress, are downregulated in the QS-deficient mutants.

Oxidative stress selects quorum quenching-resistant mutants

Next, we investigated whether QS stability is maintained due to a combination of the benefits of cooperative behavior at the population level (production of public goods) and the individual benefit of stress resistance (private goods), which may have important consequences for the bacterial populations. Recently, we demonstrated that, by imposing an adequate selective pressure, the isolation of furanone C-30-resistant mutants was possible (Maeda *et al.*, 2012). For those experiments, the pressure was exerted by growing cells with adenosine as the sole carbon source as adenosine catabolism occurs through nucleoside hydrolase, which is QS controlled (Heurlier *et al.*, 2005). Through the adenosine screening, C-30-resistant mutants were identified that have mutations in the *mexR* and *nalC* genes. Both genes encode transcriptional repressors of the *mexAB-OmpR* operon, which encodes a multidrug resistance efflux pump that we showed is able to efflux C-30 when its expression is de-repressed (Maeda *et al.*, 2012; García-Contreras *et al.*, 2013a).

In the present work, the selective pressure exerted for the enrichment of the *mexR* C-30-resistant mutants in mixed populations (wild-type and *mexR*) was oxidative stress. This stress was chosen as it is likely that *P. aeruginosa* would encounter it in the host and in natural environments. Based on the fact that the C-30-resistant *mexR* mutant was less sensitive to oxidative stress than the PA14 wild-type strain upon the addition of the furanone (Table 2B), competition experiments were done. Strains in LB were grown to a turbidity at OD 600 nm of $\sim$0.5 (note that the individual growth rates of wild-type and *mexR* mutant in LB were indistinguishable, being $1.27 \pm 0.09\,h^{-1}$ and $1.25 \pm 0.02\,h^{-1}$, respectively), were mixed in a single culture at proportions 1:1 or 1:10 (*mexR*:wild-type), and C-30 at 50 μM (or the diluents ethanol as a negative control) was added. The cells were grown until the end of exponential phase/beginning of the stationary phase (a turbidity at OD 600 nm of $\sim$1.5), and H_2O_2 (160 mM) was added; after 30 min of exposure, viable counts were done. When the strains were mixed in a 1:1 proportion, the treatment of C-30 and H_2O_2 significantly ($P<0.05$, Student's two-tailed test) enriched the *mexR* (QS^+ in the presence of C-30) mutant proportion from 0.44 ± 0.07 to 0.68 ± 0.09 (average of four independent experiments ± s.e.m.), and in contrast, treatment with ethanol and H_2O_2 decreased the proportion of the *mexR* mutant from 0.5 ± 0.08 to 0.37 ± 0.07 (Figure 2a). Hence, the Malthusian rates for the wild-type (QS^- in the presence of C-30) and *mexR* strains treated with C-30 and H_2O_2 were -0.57 and 0.44, respectively. Similarly, when strains were mixed in a 1:10 proportion, the treatment of C-30 and H_2O_2 significantly ($P<0.05$, Student's two-tailed test) enriched *mexR* proportion from 0.18 ± 0.05 to 0.35 ± 0.08 (average of four independent experiments ± s.e.m.), while in ethanol and H_2O_2 the proportion decreased from 0.17 ± 0.02 to 0.12 ± 0.05 (Figure 2b). The Malthusian rates for wild-type and *mexR* strains treated with C-30 were -0.38 and 0.66, respectively.

Furthermore, the effect of adding H_2O_2, C-30 or the combination of both of in competition experiments involving five consecutive culture passes was also investigated. For these experiments, the initial proportion of the *mexR* and wild-type strains was 1:4 (the experimentally determined proportion was 0.26 ± 0.03). Our results show that H_2O_2 alone increased *mexR* proportion slightly, to 0.38 ± 0.03 ($P<0.05$, Student's two-tailed test) and C-30 increased to 0.52 ± 0.02 after five passes ($P<0.001$, Student's two-tailed test), indicating that under these conditions, stress and QS inhibition alone could be detrimental for the wild-type cells. However, as expected, the combination treatment

including QS inhibition (C-30) and stress H_2O_2 had a much more important effect, increasing dramatically the *mexR* proportion to 0.91 ± 0.03 after five passes ($P<0.001$, Student's two-tailed test). Note that the *mexR* proportion in control cultures (when only ethanol, the C-30 diluent, was added) remained stable (0.22 ± 0.03, not statistically different from the *mexR* proportion at the beginning of the experiment; Figure 2c). These results demonstrate that mutants able to resist the QS inhibition by C-30 have better fitness upon quorum quenching/oxidative stress and higher survival than those sensitive to the inhibitor; which allows for the enrichment of QQ-resistant mutants when QQ and stress are combined.

QS control over stress response decreases social cheating

It is well known that the cooperative behavior of individual *P. aeruginosa* bacteria growing at high densities, which is triggered by the QS response, is susceptible to exploitation by social cheaters. Hence, the cheaters enjoy the benefits of the products secreted by cooperators (public goods), without devoting their own resources for the generation of such products (Diggle *et al.*, 2007). Indeed, growth of *P. aeruginosa* using protein as the sole source of carbon and energy requires QS-controlled proteases and encourages the emergence of LasR-mutant social cheaters (Diggle *et al.*, 2007; Sandoz *et al.*, 2007). Recently, it was demonstrated that the combination of a QS-controlled carbon source that is metabolized internally by the bacteria, such as adenosine (private good), and protein (public good) as carbon sources decreases the proliferation of such cheaters, preventing a 'tragedy of the commons' (Dandekar *et al.*, 2013). In addition, cheating is also diminished in a population of bacteria that are closely related (kin selection) (Diggle *et al.*, 2007; Jousset *et al.*, 2014).

In this work, we wanted to explore whether stress can also act as a counter-selective force for the appearance/survival of such QS cheaters, based on the fact that QS is involved in the development of a robust stress response against ROS, by enhancing the activity of anti-oxidative enzymes such as CAT, SOD and NADPH-producing dehyrogenases, which are private goods used only by the bacteria that produce them (the cooperators). For this experiment, protease$^-$ clones (putative) cheaters were generated by growing the PA14 wild-type strain in M9 medium, supplemented with 0.5% sodium caseinate as the sole carbon source, and sub-culturing the bacteria daily during several passes. Protease$^-$ putative QS social cheaters were detected by the lack of production of a proteolysis halo in 3% skimmed milk LB plates, which began to appear as early as pass number 4 (after $\sim$20 generations). Nine putative cheaters from different culture passes were evaluated for their production of four QS virulence factors, elastase, alkaline protease, pyocyanin and pyoverdine, and were compared with nine putative cooperative clones (positive for proteolysis halo). As expected, putative cheaters produced much less alkaline protease, elastase, pyocyanin and pyoverdine than the cooperative individuals (Figure 3).

The protease$^-$ proportion grew gradually from pass 4, and the proportion reached 26% at pass 9. At pass 9, 1 ml of the culture was stressed with H_2O_2 (160 mM) for 30 min, and 0.5 ml of the stressed culture was used to inoculate a new culture (pass 10). The proportion of protease$^-$ clones in the previously stressed cultures decreased to 17%, and in contrast, the putative cheater proportion of the non-stressed control cultures in pass 10 was $\sim$40% ($P<0.05$, $F=17.7$ ANOVA test) and remained around 45% for the following 10 passes, as previously found for PAO1-generated social cheaters (Sandoz *et al.*, 2007). The protease$^-$ proportion in the stressed cultures continued to grow after the stress but at a low rate, reaching 31% at pass 20. Moreover, if previously exposed H_2O_2 cultures were further challenged with 160 mM H_2O_2 at pass 14, the protease$^-$ proportion further decreased to 7% at pass 15 ($P<0.05$, $F=17.7$ ANOVA test) and reached only 13% by pass 20 ($P<0.05$, $F=17.7$ ANOVA test; Figure 4a). In addition, to evaluate the effect on putative cheaters' production of severe oxidative stress pulses at the middle and at three-quarters of the culture series, the effect of a moderate stress continuously administered from pass 3 to pass 20 was also evaluated (adding 40 mM or 60 mM H_2O_2 to the cultures immediately after their inoculation). The continuous administration of 40 mM H_2O_2 reduced the protease$^-$ proportion by 80% at pass 10 ($P<0.05$, $F=34$ ANOVA test), 64% at pass 15 ($P<0.05$, $F=34$ ANOVA test) and 75% at pass 20 ($P<0.05$, $F=34$ ANOVA test), while 60 mM reduced the proportion of protease$^-$ clones by 98% at pass 20 ($P<0.05$, $F=34$ ANOVA test), almost eradicating them (Figure 4b). Note that as it was shown previously with PA14 wild-type and the *lasR rhlR* mutant, the CAT and IDH activities of protease$^+$ clones increase upon H_2O_2 exposure, whereas the activities of the protease$^-$ putative cheaters decreased (Supplementary Figure S1). Therefore, these results show that oxidative stress serves to select for those cells that maintain active QS systems and that oxidative stress prevents the propagation of putative QS cheaters.

Conclusions

Our results indicate that QS enhances the stress response and that this link between QS and stress has important physiological and ecological consequences, including allowing the selection of quorum quenching-resistant mutants (Supplementary Figure S2) and reducing putative social cheaters (Supplementary Figure S3). These results could affect the bacterial population both in hosts and when it is free living

and exposed to harsh environmental conditions. In addition, the preservation of functional QS systems may be fundamental for ecological phenomena such as niche colonization, especially for those niches presenting non-optimal conditions, as it would allow bacteria to tolerate and survive better several kinds of biotic and abiotic stresses and to maintain healthy cooperative societies that limit social cheating. The constant exposure of bacteria to stress in the environment makes the QS control over the stress response a plausible mechanism that may shape and maintain functional bacterial communities in their natural environments.

Conflict of Interest

The authors declare no conflict of interest.

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