

SUPPORTING INFORMATION

Competition studies confirm two major barriers that can preclude the spread of resistance to quorum-sensing inhibitors in bacteria

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SUPPLEMENTARY TEXT.

Preparation of QS-selective media. The QS-selective medium (1, 2) (QSM; 49.3 mM Na₂HPO₄, 50.0 mM KH₂PO₄, 4.8 mM MgSO₄, 7.6 mM (NH₄)₂SO₄, 0.60 mM CaCl₂, 25 μM FeSO₄, 0.162 μM (NH₄)₆Mo₇O₂₄, 38 μM ZnSO₄, 14 μM MnCl₂, 1.6 μM CuSO₄, 0.86 μM CoCl₂, 1.9 μM BH₃O₃, 5.5 μM NiCl₂, 6.7 μM EDTA, pH 7.0) was prepared and stored as 7 QSM components (QSMCs) that were mixed and sterilized prior to use: 10× QSMC-1 (493 mM Na₂HPO₄, 500 mM KH₂PO₄, autoclaved), 100× QSMC-2 (0.483 M MgSO₄, autoclaved), 100× QSMC-3 (0.757 M (NH₄)₂SO₄, autoclaved), 500× QSMC-4 (0.299 M CaCl₂, autoclaved), 500× QSMC-5 (3.60 mM FeSO₄, sterile filtered), 5,000× QSMC-6 (0.809 mM (NH₄)₆Mo₇O₂₄, sterile filtered), 1,000× QSMC-7 (38.1 mM ZnSO₄, 18.0 mM FeSO₄, 14.2 mM MnCl₂, 1.56 mM CuSO₄, 0.861 mM CoCl₂, 1.89 mM BH₃O₃, 5.47 mM NiCl₂, 6.72 mM EDTA, pH 2.1, sterile filtered). The components were added to H₂O to dilute each up to 1×, the carbon source(s) were added (1% BSA and 0.1% CAA for group-beneficial or 0.1% adenosine for selfish), and the mixture was sterile filtered. To make solid QSM, the H₂O and an amount of agar that would give a final concentration of 1.5% was autoclaved, and the sterile components were added to the agar/water mixture after cooling to 58 °C. Aliquots of 1% BSA and 0.03% CAA were added from a 10× sterile-filtered stock immediately before pouring the plates.

Determination of non-QS-dependent, off-target growth effects of brominated furanone C-30. We initially considered performing rounds of growth in QS-selective media in the presence of small molecule QSIs to test for the development and spread of QSI-resistant mutants. However, the known QSIs of *Pseudomonas aeruginosa* either appear to have off-target effects or do not fully inhibit QS-regulated phenotypes in wild-type *P. aeruginosa*. We were concerned that one of the best reported QSIs, furanone C-30 (3), had off-target growth-inhibitory effects due to preliminary studies in our laboratory and because a very similar molecule, (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (brominated furanone BF8, Supplementary Figure

1C), was shown to have alternative non-QS-related targets in *P. aeruginosa* (4). To examine this issue, we grew *P. aeruginosa* in our QS-selective and non-QS-selective media in the presence of C-30 to test if there were concentrations of C-30 that slowed growth only under QS-selective pressure. Our experimental protocol is outlined below.

P. aeruginosa strain PA14 overnight culture was rinsed twice with 1× M9 salts by centrifugation and resuspension, and was resuspended in an equal volume of 1× M9 salts. The rinsed culture was diluted 10-fold with 1× M9 salts, and 2 µL of the dilution was added to 198 µL of various media containing furanone C-30 (Sigma-Aldrich) in a 96-well microtiter plate. The media tested were Luria-Bertani (LB, nutrient-rich), QSM + 1% CAA (minimal, non-QS-selective), and QSM + 0.1% adenosine (QS-selective). Three different concentrations of furanone C-30 were tested (15 µM, 50 µM, and 150 µM) in each medium; C-30 was added from 200× DMSO stocks, so that each well contained 0.5% DMSO, including the negative control. Three separate wells of each condition were prepared. The cultures were incubated at 37 °C with 200 rpm shaking, and OD₆₀₀ was read periodically over 30–120 h, depending on the growth medium (Supplementary Figure 1A).

Since the magnitude of shift in the growth curve depends on the general rate of growth in the medium, the faster growing media (LB and QSM + 1% CAA) appeared to have less of a shift than the slow-growing medium (QSM + 0.1% adenosine). To account for this artifact while quantifying the effects of C-30, specific growth rates (μ) were calculated for each condition and normalized to the DMSO control in each medium. To calculate specific growth rate, first the growth time required to reach an OD₆₀₀ of half the maximum OD₆₀₀ (OD_{600,mid}; indicated with a line on the plots, Supplementary Figure 1A) was calculated from the growth curve. Thereafter, the number of generations required for growth from inoculation to that OD₆₀₀ was approximated by dividing the midpoint OD₆₀₀ by the initial OD₆₀₀, taking the logarithm of that quotient, and dividing it by log(2). The average generation time was then calculated by dividing the growth time to OD_{600,mid} by the number of generations to reach OD_{600,mid}. Finally, specific growth rate (μ ,

in h^{-1}) was calculated by dividing $\ln(2)$ by this average generation time. To convert the specific growth rates to relative growth rates, each value was divided by the mean specific growth rate for the DMSO control in that medium. Each sample was treated independently for the calculations; thus, the final error bars represent a triplicate of individual relative growth rates (Supplementary Figure 1B).

Although C-30 only minimally slowed growth of *P. aeruginosa* in LB medium (corroborating a recent report by Wood and co-workers (5)), this compound inhibited the growth rate in the *non-QS-selective* minimal medium (CAA) by approximately 25% at concentrations as low as 15 μM . Further, the degree of growth inhibition in the QS-selective medium (adenosine) was *no greater* than the degree of growth inhibition in the non-QS-selective medium (CAA; Supplementary Figure 1B)—indicating that the growth inhibitory effect of C-30 is not primarily due to its QS-inhibitory activity. The QSM + 1% CAA conditions were repeated on a separate day with both *P. aeruginosa* strains PA14 and PAO1, and similar growth effects were caused by C-30. Since these off-target growth effects would certainly cause a non-QS-based selective pressure for resistance under our experimental conditions, we chose not to use C-30 as a QSI in the current study. As stated in the main text, other reported QSIs do not inhibit elastase B production substantially. For example, two of the most potent reported *P. aeruginosa* QSIs that act *via* the LasR receptor (V-06-018 (6) and *N*-(4-bromophenylacetanoyl)-L-homoserine lactone (7)) cannot inhibit elastase B production in wild-type *P. aeruginosa* PAO1 by greater than 60%, which would provide too weak of a QS-dependent selective pressure to obtain unambiguous results. We therefore developed a model system using mutant *P. aeruginosa* strains that mimics resistance development to an ideal QSI that inhibits QS at nearly 100% with no off-target effects (see main text and Figure 3 for a detailed schematic of our model).

Construction of QSI-resistant (R) and QSI-sensitive (S) mimic strains. The QSI-resistant mimic strain (R) (GFP^+ , Gm^{R}) and the QSI-sensitive mimic strain (S) (ΔlasR , ΔrhIR , Tc^{R}) were

constructed from the same parent *P. aeruginosa* PAO1 strain to avoid any fitness differences due to microevolution (8). To construct the gentamicin-resistant “QSI-resistant mimic” strain, a mini-Tn7 transposon harboring GFP and *aaaC1* (i.e., gentamicin resistance, Gm^R) was inserted into the neutral *attTn7* site of the PAO1 chromosome by 4-parental mating between *P. aeruginosa* PAO1 and *E. coli* strains harboring pUXBF-13 (9), pRK600 (10), and pMiniTn7-gfp2 (11), followed by citric acid selection on Vogel-Bonner (VB) minimal medium (57.4 mM K₂HPO₄, 8.37 mM NaNH₄HPO₄, 0.811 mM MgSO₄, 9.52 mM citric acid (12)) + gentamicin (13). Single colonies were restreaked on VB + gentamicin, and an individual colony from the second plate was picked (named strain PAO1-Tn7-gfp/Gm^R, also called “R”) and verified for Tn7 insertion both by observing GFP fluorescence and by colony PCR with primers Tn7-GlmS (5'–AATCTGGCCAAGTCGGTGAC–3') and Tn7R109 (5'–CAGCATAACTGGACTGATTTTCAG–3') (11).

The same parental PAO1 strain was mutated by homologous recombination to construct the tetracycline-resistant “QSI-sensitive mimic” strain. To replace the *lasR* gene with a tetracycline resistance (Tc^R) cassette, an approximately 5 kb Δ *lasR*::Tc^R cassette was amplified from PAO-JP3 (14) genomic DNA with primers D060 (5'–GCTGCTCGGCTTCTGGGTG–3') and D061 (5'–ACGTTTGCCCCGCTACTGG–3'). The amplified region contained a *KpnI* site and an *EcoRI* site that were used to clone into *KpnI/EcoRI*-cut pEX18Gm (15). The resulting plasmid pJG038 was transformed into *E. coli* S17-1 λ pir by electroporation and then transferred into PAO1 by conjugation. Individual merodiploid colonies that were isolated by selection on VB + gentamicin plates were picked and selected for the second recombination event by growth on LB + 15% sucrose at 30 °C. Strain PAO-JG33 was isolated, and sequencing of DNA amplified from its *lasR* region confirmed the gene deletion. PAO-JG33 produced significantly less green pigment than wild-type PAO1 and displayed virtually no ability to degrade an elastin congo red substrate compared to wild-type PAO1 (using a previously reported method (7); Supplementary Figure 2A).

We next deleted the *rhIR* gene from PAO-JG33 because others have shown that *rhIR* can compensate for *lasR* mutants (16, 17). To delete the *rhIR* gene, an approximately 1 kb upstream portion was amplified from PAO1 genomic DNA with primers D062 (5'-ACTGGATTCCAACGTGCCCGAGCAG-3') and D063 (5'-ACTTCTAGATGCAGTAAGCCCTGATCGATAAAATG-3'), and an approximately 900 bp downstream portion was amplified from PAO1 genomic DNA with primers D083 (5'-ACTTCTAGAACTTCCACCACAAGAACATCCAGA-3') and D066 (5'-ACTAAGCTTCCAAATCCCGGAATGCAGG-3'). The two pieces were cut with *XbaI*, ligated together, reamplified with primers D062 and D066, digested with *BamHI* and *HindIII*, and ligated into *BamHI/HindIII*-cut pEX18Gm. The resulting plasmid pJG055 was introduced into PAO-JG33 via the same mating method as above for pJG038. After selection on VB + gentamicin and counter-selection on LB + sucrose, strain PAO-JG35 was isolated. Sequencing of DNA amplified from its *rhIR* region confirmed the gene deletion. PAO-JG35 cleaved even less elastin congo red substrate than PAO-JG33 (Supplementary Figure 2A). PAO-JG35 (also called "S") did not grow differently from PAO1 or PAO1-Tn7-gfp/Gm^R (or "R") in non-QS-selective media (Supplementary Figure 2B), but did grow significantly slower than the R strain in QS-selective media (see Figures 1A and 1B in main text). For microscopy, pMP7605 (18) was conjugated into strain S by mating with *E. coli* S17-1 λ pir containing pMP7605, followed by selection on VB + gentamicin.

Construction of a "signal-independent" constitutive *lasB*-producing QSI-resistant (R-*lasB*) mimic strain. The P_{tac} -*lasB* cassette from pML27 (19) was amplified with primers D108 (5'-CAGGCTGAAAATCTTCTCTCATCC-3') and D110 (5'-AAA ACTTAAGCCTGAACTTTAGACCGGGTTC-3'). The amplified DNA was cut with *KpnI* and *AflIII*. Likewise, pMP7607 (18) was cut with *KpnI* and *AflIII*, which removed the mCherry gene. The cut P_{tac} -*lasB* insert was ligated into the cut pMP7607 to yield pJG068. The mini-Tn7

cassette harboring P_{tac} -*lasB* was inserted into the PAO1 genome via the same procedure as for adding the GFP-Gm^R cassette to construct strain R above, except that Pseudomonas isolation agar (PIA, 2% peptone, 0.69 mM MgCl₂, 57 mM K₂SO₄, 1.36% agar, 2% glycerol, 25 mg/mL irgasan) + streptomycin was used for selection instead of VB + gentamicin. The procedure was carried out on both wild-type PAO1 and PAO-JG35 to obtain PAO1-Tn7-*lasB* (termed R-*lasB*) and PAO-JG35-Tn7-*lasB* (termed S-*lasB*). R-*lasB* had greater protease activity than strain R, and S-*lasB* had much greater protease activity than strain S (Supplementary Figure 5A), confirming insertion. Insertion was also confirmed by colony PCR with primers Tn7-GlmS (5'-AATCTGGCCAAGTCGGTGAC-3') and Tn7R109 (5'-CAGCATAACTGGACTGATTTTCAG-3') (11).

Plating different degrees of population structure on solid QS-selective media. Plates were inoculated three different ways to afford different degrees of population structure. To make a finely mixed population, sterile glass beads were used to spread 100 μ L of a 10² \times dilution of the rinsed mixed R/S culture. The 10² \times dilution deposited individual bacteria that were approximately 0.1 mm apart from each other, which grew into 0.1 mm diameter microcolonies by metabolizing the 0.03% CAA carbon source. To make an intermediately mixed population, a 10⁴ \times dilution of the rinsed mixed R/S culture was used; this led to more sparsely deposited bacteria that grew to form 1 mm diameter microcolonies after CAA digestion. Finally, to examine the least degree of mixing, 1 μ L aliquots of rinsed R and S monocultures were individually spotted approximately 1 cm apart on a grid that filled the 10 cm diameter plate with 100 spots (see Figures 4B, 4C, and 4D in main text for images of different degrees of population structure). The individual spotting was required to ensure that each plate had one resistant patch and 99 sensitive patches (approximately 1:100 R/S ratio)—a spreading technique similar to the others but using a 10⁶ \times dilution would be susceptible to many random instances of either

zero resistant bacteria being present or more than a single resistant patch being present (> 1:100 R/S ratio).

Microscopy. Bacterial population structure was imaged on a Zeiss AX10 Imager.M2 epifluorescent microscope with a HXP 120 C Lamp using the 2.5×/0.12 FLUAR objective in conjunction with an AxioCam MR monochrome camera controlled by AxioVision (Rel 4.8.2) software (Carl Zeiss MicroImaging). GFP and mCherry filters were used. Samples were prepared identically to those for solid competition experiments except that the S strain harbored pMP7605 (18). Microscopy was performed directly through the agar plate after incubation at 30 °C for 6 days. The microscope's field of view was not sufficiently large to display several microcolonies, so nine partially overlapping images were taken with the same exposure settings and overlaid without modification using Adobe® Photoshop. The composite images were cropped to have flush edges and to be of the same size.

Quantification of OdDHL produced by mixtures of R and S strains. To verify that non-quorate levels of the *P. aeruginosa* OdDHL signal molecule were present with R/S ratios ≤ 1:100, OdDHL was extracted from QSM + 0.1% CAA cultures upon reaching stationary phase and quantified using a bioreporter assay (Supplementary Figure 3). The bioreporter assay quantified OdDHL by measuring LasR-activated GFP production by the heterologous reporter *E. coli* JLD21/pJN105L + pPROBE-KL (20). Inoculation and growth were performed as described in the Methods section of the main text, except 5 µL aliquots of undiluted rinsed cultures were used to inoculate 5 mL portions of media in 14 mL culture tubes. After 10 h of growth, the cultures reached stationary phase, and they were pelleted. An unincubated 2 µM control was also prepared, where 4 mL of medium (without bacteria) was treated with 2 µM OdDHL from a 4 mM DMSO stock.

A 4-mL aliquot of culture supernatant or control was extracted with 3 × 2 mL ethyl acetate, and the ethyl acetate was evaporated. Each isolated residue was redissolved in 300 μ L LB + gentamicin + kanamycin and serially diluted by factors of 3 into LB + gentamicin + kanamycin. A 180 μ L portion of these dilutions was added to 20 μ L portions of the *E. coli* JLD21/pJN105L + pPROBE-KL bioreporter that had been grown overnight in LB + gentamicin + kanamycin and added to 96-well black-walled, clear-bottom microtiter plates. Arabinose was added to the mixtures at a final concentration of 0.4% to induce LasR production. These bioreporter cultures were incubated at 37 °C with 200 rpm shaking for 7 h. Fluorescence (λ_{ex} : 500/27 nm, λ_{em} : 540/25 nm) was read using a plate reader and normalized by OD₆₀₀. Dilution-response curves were obtained (Supplementary Figure 3A), and the concentrations of OdDHL present in the QSM + 0.1% CAA cultures were approximated by observing the curve shifts compared to the 2 μ M control (Supplementary Figure 3B). The 1:100 R/S mixture produced approximately 300 pM OdDHL, which was nearly 1000 \times less than wild-type PAO1 and well below the quorate concentration (Supplementary Figure 3B). These results strongly suggest that rare R (\leq 1%) should not be capable of expressing its QS regulon under the conditions tested in this study.

Degradation of OdDHL in *P. aeruginosa* cultures. To mimic “signal-independent” QSI resistance, we first tried to induce QS in the R strain when it was rare by the exogenous addition of 2 μ M OdDHL. We were concerned, however, that OdDHL might degrade in the media during the course of the experiment. Although previous reports indicate that OdDHL is fairly stable to non-enzymatic lactonolysis in buffered media (21, 22), this signal remains susceptible to enzymatic degradation by the many acylases produced by *P. aeruginosa* (23-25). If these enzymes degrade OdDHL substantially within hours, then the exogenous addition of OdDHL potentially would not be sufficient to activate QS-regulon expression when R is rare. We tested the extent to which exogenously added OdDHL was degraded by the bacteria under our growth

conditions (in 0.1% CAA to mimic the initial stages of growth in both 1% BSA + 0.1% CAA, and in 0.1% adenosine). The experimental protocol was the same as that for quantifying production of OdDHL outlined above, except that 2 μ M OdDHL was exogenously added to each sample from a 4 mM stock solution (final DMSO concentration 0.1%) prior to incubation. An additional control of OdDHL incubated at 37 °C in the growth medium without bacteria was included to measure non-enzymatic lactonolysis (i.e., background hydrolysis). As above, the final dilution-response curves were compared to the dilution-response curve of a 2 μ M standard (Supplementary Figures 4A and 4C), and concentrations of OdDHL remaining after incubation were approximated by the shift observed relative to the 2 μ M standard (Supplementary Figures 4B and 4D).

After 10 h of incubation in the CAA medium, nearly no background hydrolysis of OdDHL had occurred (by analysis of the “no bacteria” control), but growth with *P. aeruginosa* caused substantial degradation (Supplementary Figure 4B). The 1:100 R/S culture had only 1% of the initial OdDHL remaining (20 nM). This level of degradation was not observed in the adenosine medium with *P. aeruginosa*; 30% of the OdDHL remained after an entire day, which was very similar to the level of background hydrolysis after one day (Supplementary Figure 4D). At this rate, multiple days would be required for OdDHL to degrade below quorate levels in the adenosine medium. Therefore, these results indicate that exogenous addition of OdDHL is a viable strategy for mimicking “signal-independent” resistance in the selfish adenosine selective medium. However, since OdDHL is 99% degraded within hours in the group-beneficial BSA selective medium, QS cannot be induced reliably via exogenous OdDHL addition in this medium. Instead, the R-*lasB* strain was used, which constitutively expresses *lasB* under a *tac* promoter without the need of OdDHL (see below).

Confirmation of active protease secretion by non-quorate R-*lasB*. Others have reported that the *P. aeruginosa* *xcp* protein secretion machinery is required to secrete active LasB, and

that *xcp* expression is also regulated by QS (26, 27). Therefore, we reasoned at the outset that constitutive expression of *lasB* under a *tac* promoter could possibly not translate to secretion of active LasB under non-quorate conditions due to lack of *xcp* expression. However, we found that that active LasB was indeed secreted from P_{tac} -*lasB* cells under non-quorate conditions by testing supernatant for ability to digest a LasB substrate (azocasein) (28, 29), as described below.

To compare protease production by strains R and S to strains R-*lasB* and S-*lasB*, cultures were grown in QSM + 0.1% CAA in 96-well microtiter plates (200 μ L in each well) for 20 h at 37 °C with shaking at 200 rpm. The cells were pelleted, and 25 μ L of the supernatant was added to 75 μ L of 1% azocasein (Sigma-Aldrich) in buffer (50 mM Tris, pH 7.8). The digestion was incubated for 5 h at 37 °C, and remaining azocasein was precipitated with 100 μ L of 15% trichloroacetic acid (TCA) in H₂O. After centrifuging at 16,000 \times *g* for 10 min, 109 μ L of supernatant was added to 91 μ L of 1 M NaOH, and absorbance (λ = 440 nm) was read using a plate reader. Absorbance of a negative control (digestion by 1 \times M9 salts) was subtracted from each sample. The P_{tac} -*lasB* cassette induced production of active LasB even in the QS⁻ (Δ *lasR*, Δ *rhIR*) S-*lasB* strain for both replicates tested (Supplementary Figure 5A), demonstrating that active QS is not needed to secrete active LasB from our “signal-independent” system.

We also tested the ability of rare R-*lasB* to produce near quorate levels of active LasB by repeating the protease assay, but with 1:100 R/S (non-quorate negative control), 1:100 R-*lasB*/S (non-quorate, constitutive *lasB*), and 1:1 R/S (quorate positive control). Since less overall protease should be produced by the 1:100 mixtures (due to only 1% of the cells being capable of producing protease when quorate compared to 50% in the 1:1 mixture), 5 \times 200 μ L of each 1:100 culture were grown. The supernatants were pooled, and 500 μ L was concentrated 20-fold to approximately 25 μ L with a 10 kDa MW cut-off spin filter (Millipore Amicon Ultra – 0.5 mL) before addition to azocasein and incubation as described above. CFUs were also determined from the final cultures. The final protease activity was normalized by both the degree of

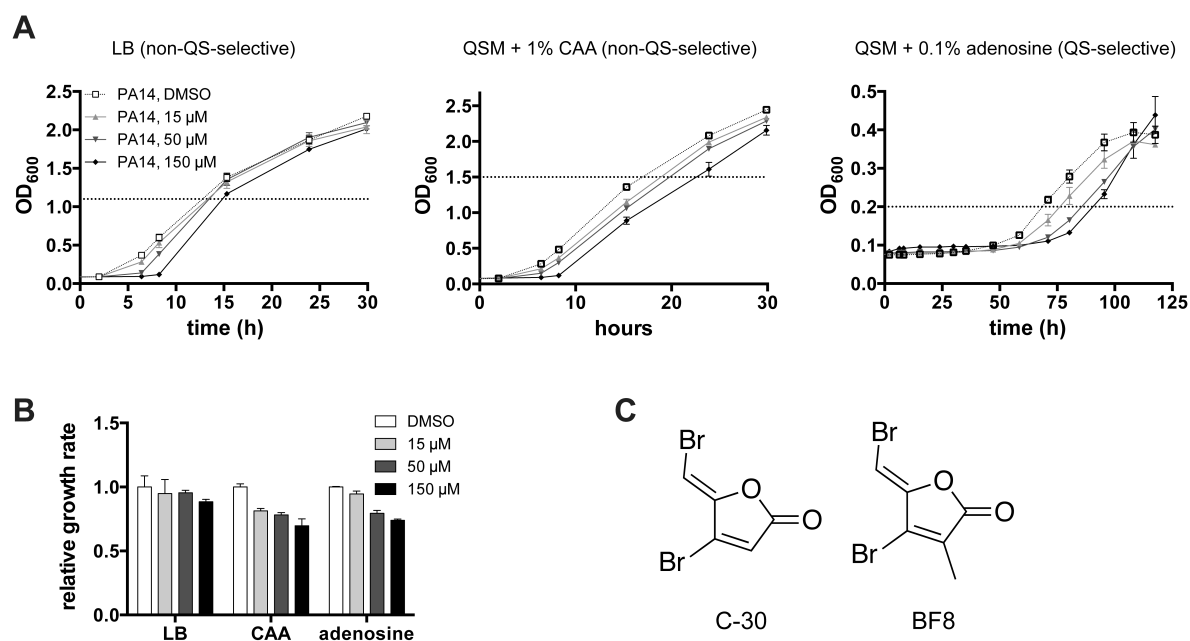
supernatant concentration used (20-fold concentration vs. no concentration) and the ratio of R/S (or R-*lasB*/S) in the culture to obtain the protease activity produced per R (or R-*lasB*) cell. Data are represented as percentage of digestion by the 1:1 R/S positive control (Supplementary Figure 5B). The 1:100 R-*lasB*/S culture produced significantly more protease than the 1:100 R/S culture, and nearly as much protease (per R cell) as the 1:1 R/S positive control. These data confirm that P_{tac} -*lasB* indeed enables the “signal-independent” QSI-resistant mimic to secrete active LasB, even in the absence of quorate signal levels.

Supplementary Table 1. Bacterial strains and plasmids used in this study.

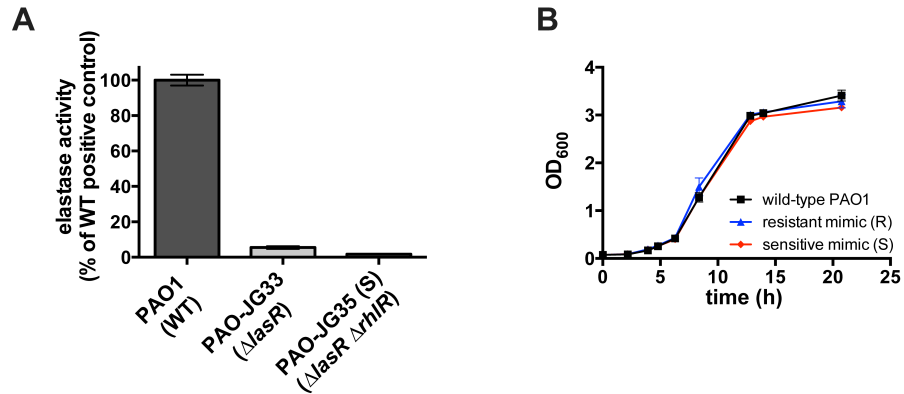
Strain or plasmid	Description*	Reference
<i>E. coli</i>		
DH5 α	F ⁻ , j80d/lacZDM15D(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rk ⁻ , mk ⁺) <i>phoA supE44 λ thi-1 gyrA96 relA1</i>	Invitrogen
S17-1 λ pir	<i>recA pro thi hsdR⁻ hsdM⁺</i> RP4-2-Tc::Mu-Km::Tn7 λ pir; Sm ^R Tp ^R	(30)
JLD-271	K-12 Δ <i>lacX74 sdiA271::Cam</i> ; Cm ^R	(31)
<i>P. aeruginosa</i>		
PAO1	Wild-type, isolated by B. Holloway from human wound	(32)
PA14	Wild-type, isolated by M. Schroth from human wound	(33)
PAO-JP3	PAO1 Δ <i>lasR::Tc^R rhIR::Tn501</i> ; Tc ^R Hg ^R	(14)
PAO1-Tn7-gfp/Gm ^R	PAO1 with mini-Tn7-based insertion of constitutive GFP Mut3b expression behind a P _{A1/04/03} promoter; QSI-resistant mimic R; Gm ^R	This study
PAO-JG33	PAO1 Δ <i>lasR::Tc^R</i> ; Tc ^R	This study
PAO-JG35	PAO1 Δ <i>lasR::Tc^R ΔrhIR</i> ; QSI-sensitive mimic S; Tc ^R	This study
PAO1-Tn7- <i>lasB</i>	PAO1 with mini-Tn7-based insertion of constitutive <i>lasB</i> expression behind a <i>tac</i> promoter; R- <i>lasB</i> ; Sm ^R	This study
PAO-JG35-Tn7- <i>lasB</i>	PAO-JG35 with mini-Tn7-based insertion of constitutive <i>lasB</i> expression behind a <i>tac</i> promoter; S- <i>lasB</i> ; Sm ^R	This study
<i>Plasmids</i>		
pUX-BF13	Mini-Tn7 helper plasmid; Ap ^R	(9)
pRK600	Conjugation helper plasmid; Cm ^R	(10)
pBK-miniTn7-gfp2	Mini-Tn7 plasmid expressing GFP Mut3b & Gm ^R ; Ap ^R	(11)
pEX18-Gm	Gene-replacement vector; <i>sacB oriT</i> Gm ^R	(15)
pJG038	pEX18-Gm with Δ <i>lasR::Tc^R</i> cassette	This study
pJG055	pEX18-Gm with markerless Δ <i>rhIR</i> cassette	This study
pMP7605	pBBR1 MCS-5 derivative expressing the mCherry gene under a <i>tac</i> promoter, Gm ^R	(18)
pMP7607	pBK-miniTn7-Km Ω Sm1 derivative expressing the mCherry gene under a <i>tac</i> promoter, Ap ^R	(18)
pML27	P _{<i>tac</i>} - <i>lasB</i> expression plasmid; <i>lacIq</i> Ap ^R	(19)
pJG068	pBK-miniTn7-Km Ω Sm1 derivative expressing <i>lasB</i> under a <i>tac</i> promoter, Ap ^R	This study
pJN105L	Arabinose-inducible expression plasmid for <i>lasR</i> ; Gm ^R	(34)
pPROBE-KL	<i>lasI'-gfp[LVA]</i> transcriptional fusion derivative of pPROBE-KT(35); Km ^R	(20)

*Abbreviations: Sm^R, streptomycin resistance; Tp^R, trimethoprim resistance; Cm^R, chloramphenicol resistance; Tc^R, tetracycline resistance; Gm^R, gentamicin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance.

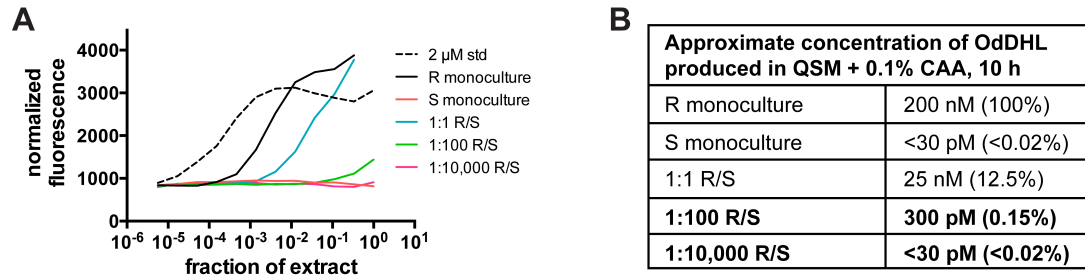
SUPPLEMENTARY FIGURES & LEGENDS.



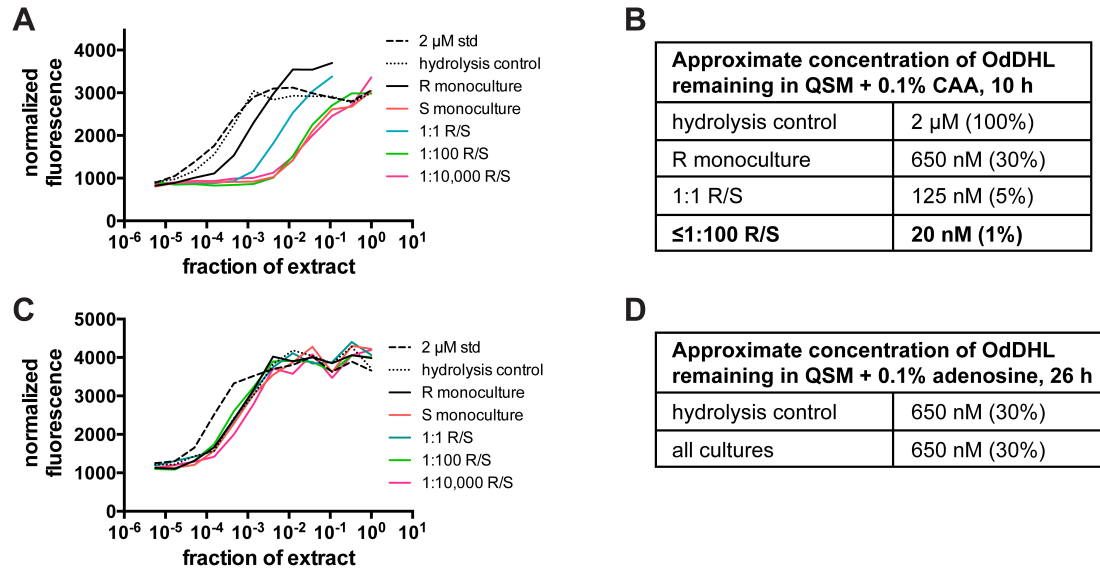
Supplementary Figure 1. Growth inhibitory effects of brominated furanone C-30 on *P. aeruginosa* PA14. **(A)** Growth curves of PA14 in LB, QSM + 1% CAA (non-QS-selective minimal medium), and QSM + 0.1% adenosine (QS-selective minimal medium) with increasing concentrations of C-30. Error bars represent s.e.m. ($n = 3$). Dashed lines indicate the half-maximal OD₆₀₀ (OD_{600, mid}) used for calculating average growth rates. **(B)** Average growth rates of PA14 with increasing concentrations of C-30. Values are normalized to the average PA14 growth rate in that medium with no C-30 added. Error bars represent s.e.m. ($n = 3$). C-30 clearly slowed the growth rate of *P. aeruginosa* by ~25% not only in the QS-selective medium (adenosine), but also in a comparable *non-QS-selective* medium (CAA)—indicating that the growth inhibitory effect of C-30 is not primarily due to its QS-inhibitory activity. **(C)** The chemical structures of brominated furanones C-30 and BF8 (another known QS modulator in *P. aeruginosa*) are shown for comparison. BF8 is known to have non-QS targets in *P. aeruginosa* (4).



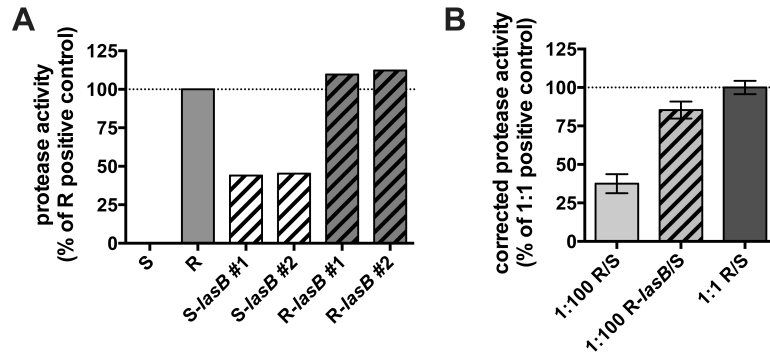
Supplementary Figure 2. Elastase activity and growth curves of wild-type *P. aeruginosa* PAO1 compared to mutants. **(A)** Elastase activity quantified by digestion of an elastin congo red substrate. Values were normalized to the activity of wild-type PAO1. **(B)** Growth curves for R and S strains and wild-type PAO1 in QSM + 1% CAA (non-QS-selective minimal medium). No fitness difference was observed over the 20 h growth period. Error bars in both plots represent s.e.m. (n = 3).



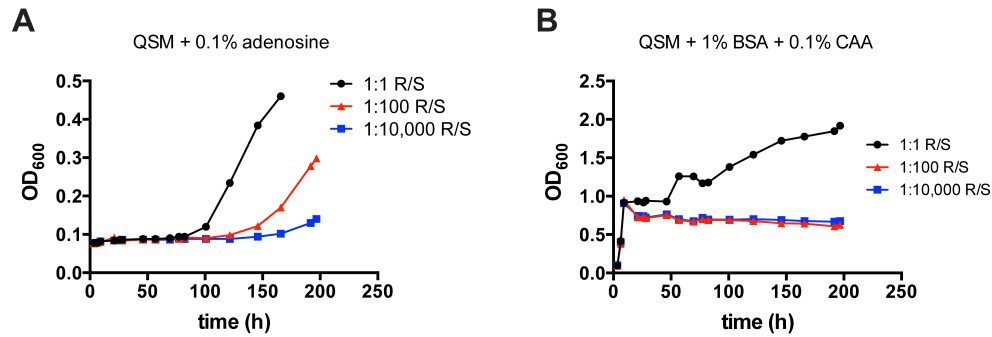
Supplementary Figure 3. Quantification of the native *P. aeruginosa* QS signal, OdDHL, in mixed R/S cultures. OdDHL was extracted from QSM + 0.1% CAA cultures grown for 10 h and serially diluted for quantification by using a LasR bioreporter assay. **(A)** Dilution-response curves for extracts of various bacterial cultures showing fluorescence of the LasR bioreporter strain relative to its OD₆₀₀. The standard (std) curve was 2 μM OdDHL that was not incubated with bacteria; the other curves were for 2 μM OdDHL incubated with bacterial cultures. **(B)** The amount of OdDHL produced by each culture (approximated based on the amount of dilution required for the 2 μM standard to give comparable activity to the extracts). Ratios ≤ 1:100 R/S produced << 1% of as much OdDHL as wild-type *P. aeruginosa*, which should not be sufficient for QS-regulated gene expression over the conditions tested in the current study.



Supplementary Figure 4. Degradation of OdDHL by *P. aeruginosa* cultures. OdDHL was extracted from cultures that initially had 2 μ M OdDHL added prior to incubation with bacteria. The OdDHL extracts were diluted and quantified by calculating the degree of dilution required of the 2 μ M OdDHL standard to reach the same activity as the extract. **(A–B)** OdDHL extracted from QSM + 0.1% CAA cultures grown for 10 h. Cultures of 1:100 and 1:10,000 R/S readily degraded OdDHL to 1% of its initial concentration after only 10 h. **(C–D)** OdDHL extracted from QSM + 0.1% adenosine cultures grown for 26 h. All adenosine cultures lost OdDHL at a much slower rate than the CAA cultures. The OdDHL degradation in adenosine medium was presumed to be mostly due to non-enzymatic hydrolysis because the remaining levels of OdDHL in this medium were indistinguishable from the level of OdDHL in the no bacteria control.



Supplementary Figure 5. Confirmation of LasB production by the “signal-independent” R-*lasB* mutant. **(A)** Plot comparing protease activity (azocasein digestion) of supernatants from bacterial monocultures grown in QSM + 0.1% CAA. Each bar is a single trial with one replicate (#1 and #2 represent two trials with the same strain). Protease activities were normalized to the positive control (strain R). S-*lasB* produced nearly half the protease activity as the R positive control in monoculture, indicating that QS is not essential for the secretion of moderate levels of active LasB when it is expressed heterologously under P_{tac} . **(B)** Plot comparing azocasein digestion of supernatants of mixed bacterial cultures grown in QSM + 0.1% CAA. Protease activities were corrected for the percentage of R (or R-*lasB*) cells present and normalized to the “quorate” positive control (1:1 R/S). Non-quorate levels of R-*lasB* (1:100 R-*lasB*/S) produced nearly as much protease per R cell as the quorate 1:1 R/S positive control, confirming that P_{tac} -*lasB* induces expression of active LasB under non-quorate, rare R conditions. Error bars represent s.e.m. (n = 3).



Supplementary Figure 6. Representative bacterial growth curves from competition experiments. **(A)** Growth curves of R/S co-cultures in the selfish QSM + 0.1% adenosine QS-selective medium. **(B)** Growth curves of R/S co-cultures in the group-beneficial QSM + 1% BSA + 0.1% CAA QS-selective medium. As expected, slower growth is observed in both media when R is present at a lower frequency. Theoretically, selective growth could potentially still occur even when no overall growth is apparent, as an increase in the growth of rare R bacteria could be “masked” by a lack of growth by the larger S population.

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