SUPPLEMENTARY MATERIAL

Pseudomonas aeruginosa reaches collective decisions via transient segregation of quorum sensing activities across cells

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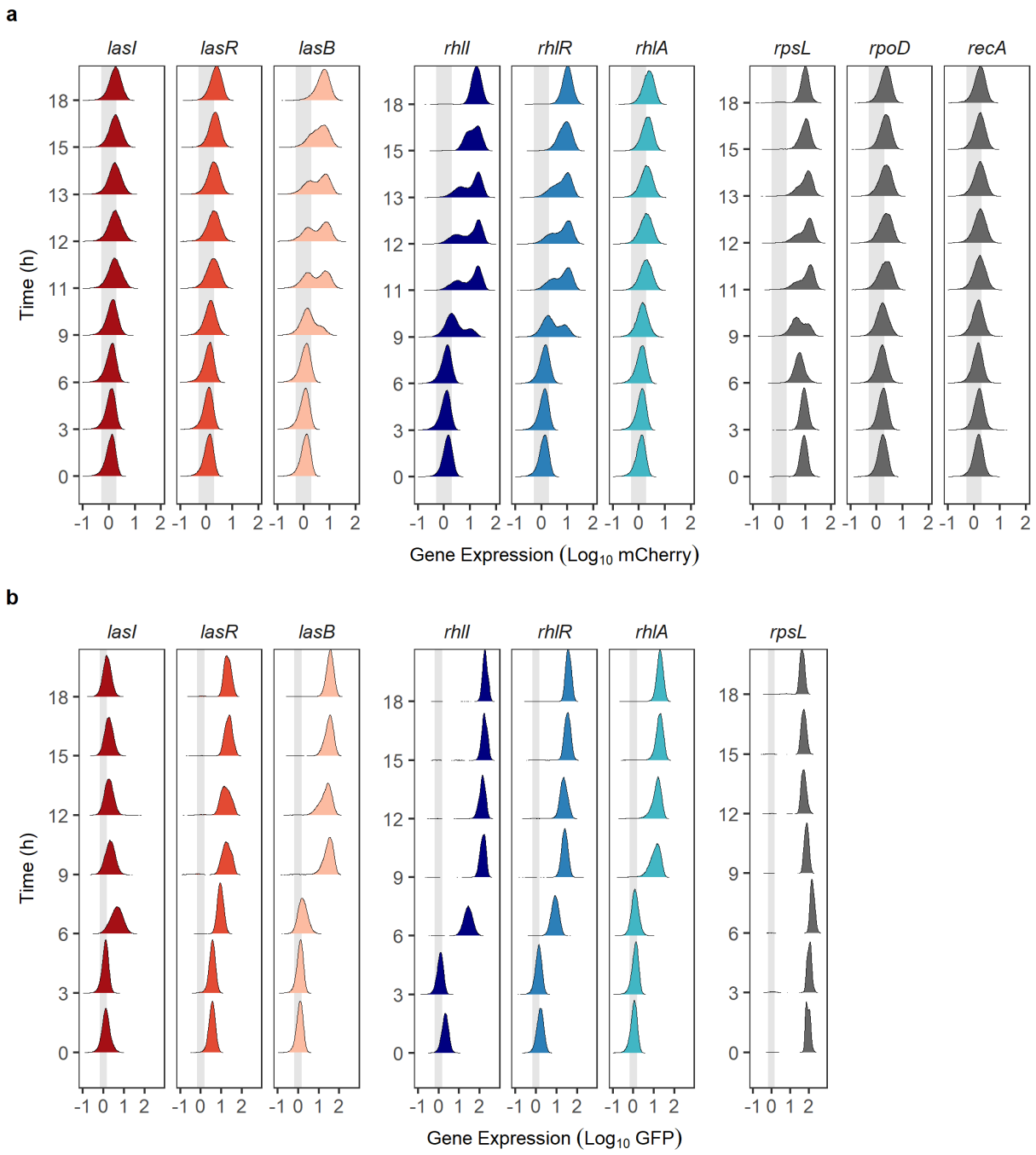
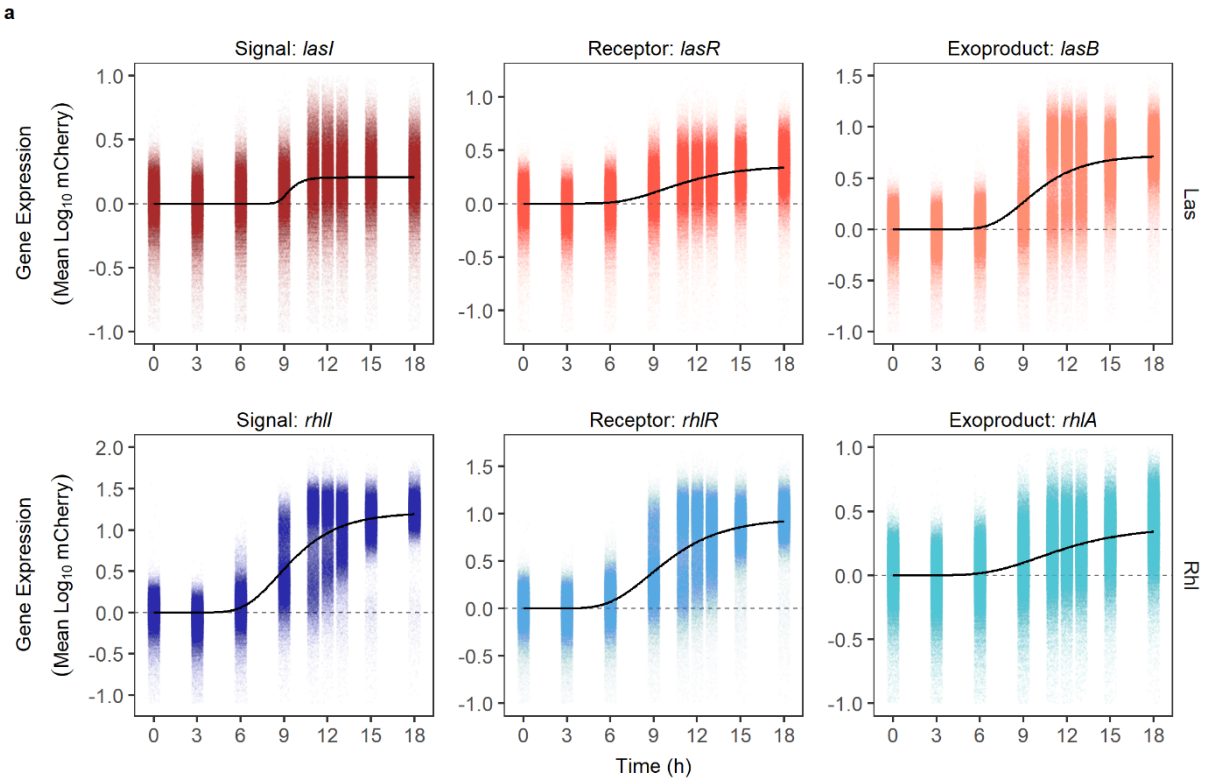
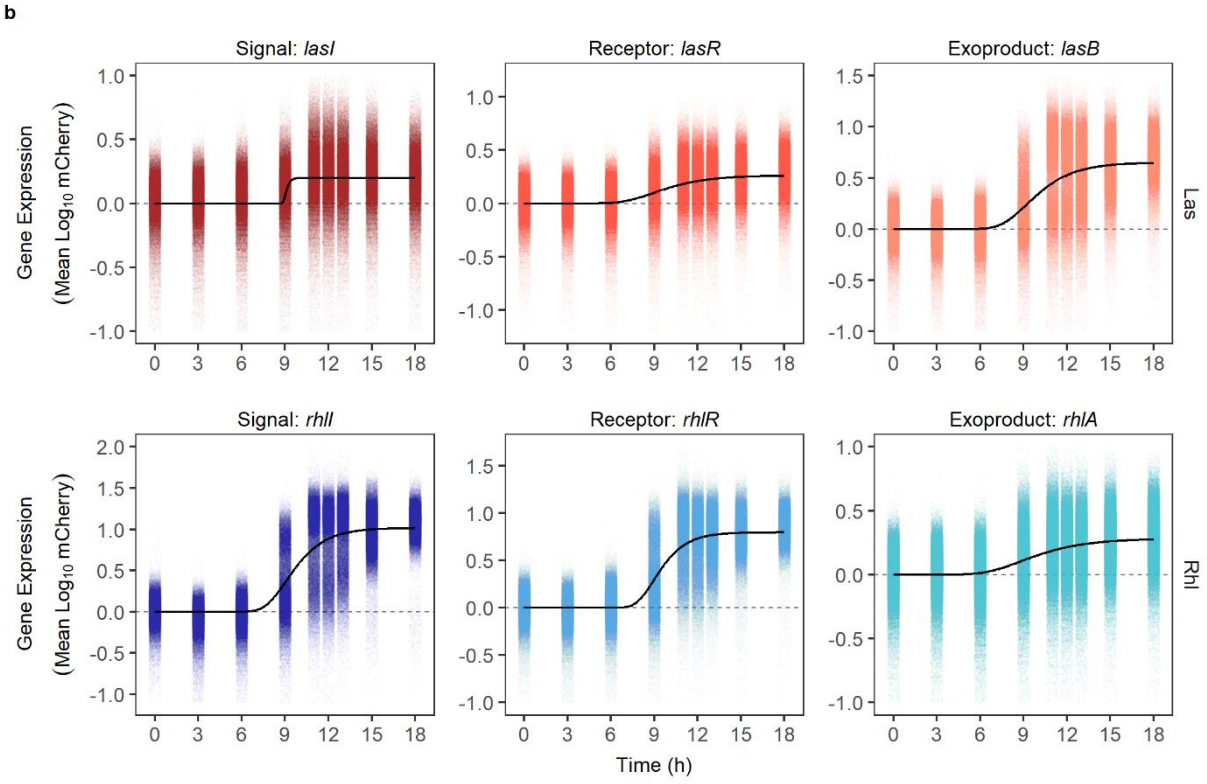
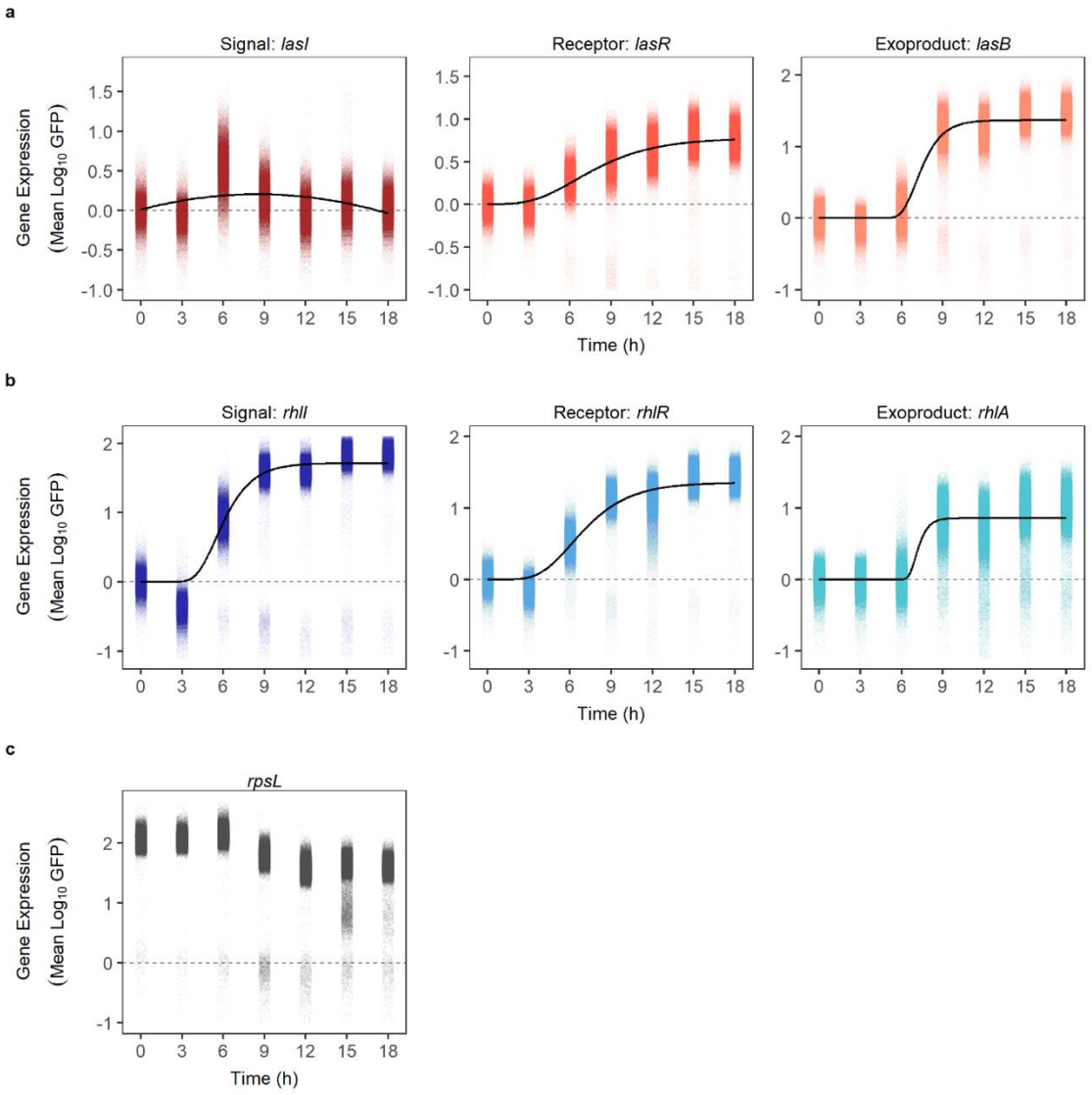


Supplementary Figure 1. Distribution of QS gene expression across single cells. Gene expression distribution of *las, rhl* and housekeeping genes when fused to mCherry (a) or GFP (b) in wild type PAO1 over an 18-hour growth cycle in LB medium. Distribution represents 150,000 cells per time point. Data stems from three independent replicates (i.e. Fig. 3 and Supplementary Fig. 2). Grey shaded area represents the standard deviation of background fluorescence.

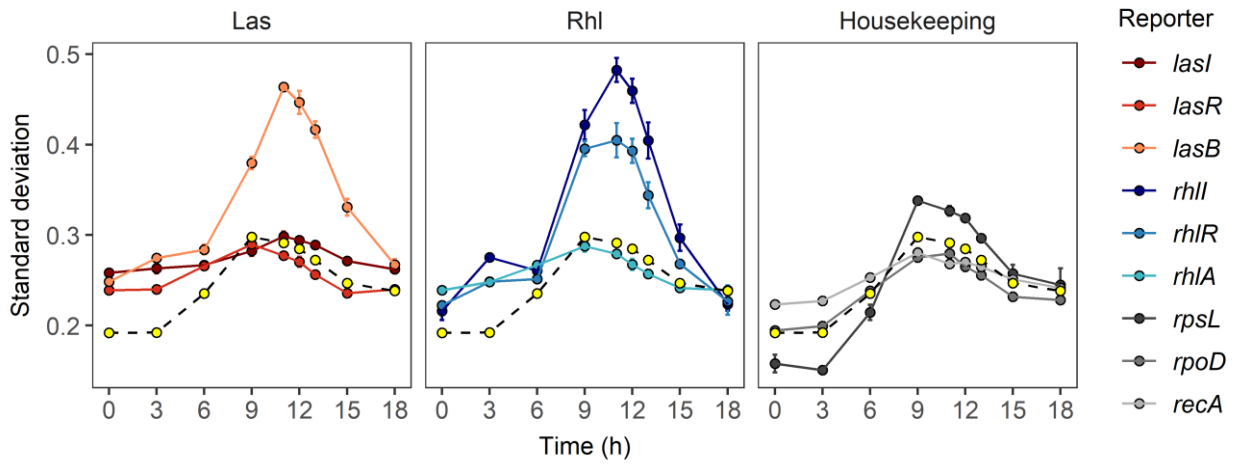

a
Signal: lasI
Receptor: lasR
Exoproduct: lasB
Signal: rhlI
Receptor: rhlR
Exoproduct: rhlA
Gene Expression (Mean Log10 mCherry)
Las
Rhl
Time (h)


b
Signal: lasI
Receptor: lasR
Exoproduct: lasB
Signal: rhlI
Receptor: rhlR
Exoproduct: rhlA
Gene Expression (Mean Log10 mCherry)
Las
Rhl
Time (h)

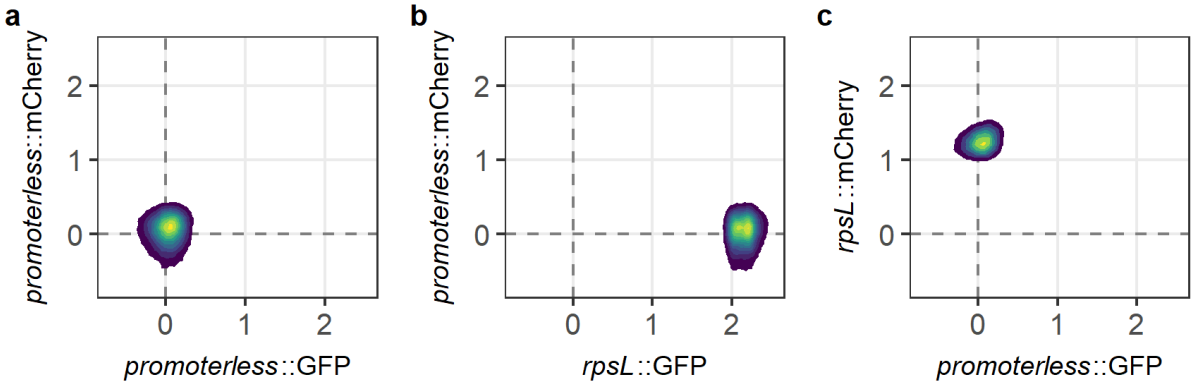
Supplementary Figure 2. Repeats of single-cell QS gene expression patterns over time. Single-cell expression trajectories of *las* and *rhl* genes for repeat 2 (a) and repeat 3 (b). See Fig. 3 for Repeat 1. Each dot represents a single cell (N = 50,000 per time point) detected and measured with flow cytometry. Dashed line at y = 0 represent the threshold value below which there is no measurable gene expression activity. Black lines show the best model fit (Gompertz function) capturing the temporal dynamics of single-cell gene expression.



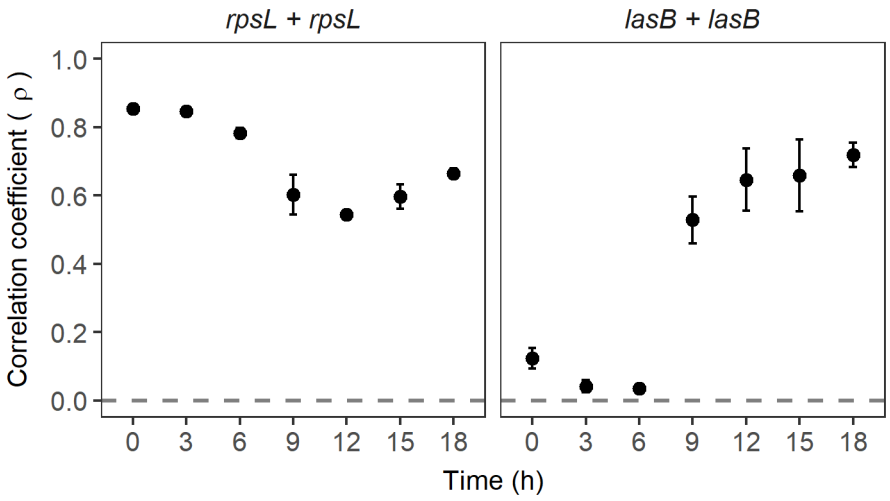
Supplementary Figure 3. Single-cell expression of GFP-tagged QS genes. Single-cell expression of *las* (a), *rhl* (b) and housekeeping gene, *rpsL* (c) in wild type PAO1 cells when fused with GFP in the chromosomally integrated double fluorescent gene reporters (in comparison to single mCherry reporters in Figure 3). Each dot represents a single cell (N = 50,000 per time point) detected and measured with flow cytometry. Gene expression has been background subtracted by the fluorescence of the non-fluorescent wild type strain. Dashed line at y = 0 represent the threshold value below which there is no measurable gene expression activity. Black lines show the best model fit capturing the temporal dynamics of single-cell gene expression (Gompertz function for all QS genes, except *lasI* for which a quadratic function explained most of the variation). Because of differences in the reporter strengths, the y-axis scale varies across panels. The figure shows representative data from one out of three complete experimental repeats.



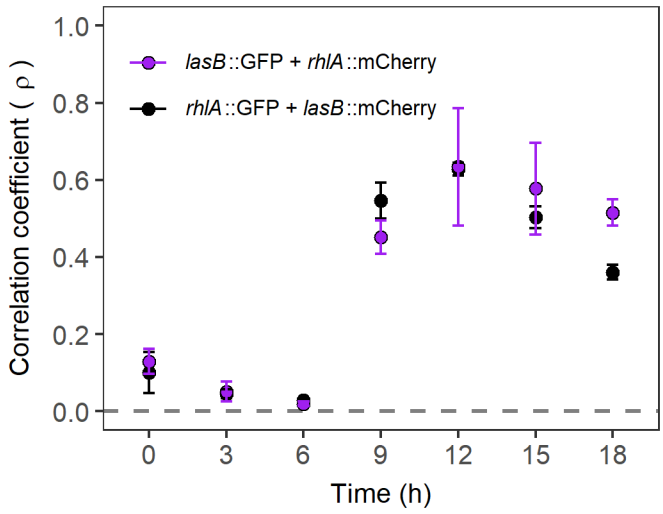
Supplementary Figure 4. Comparing standard deviations (SD) across cells shows that QS gene expression is more heterogenous than housekeeping gene expression. Panels show the SD of las, *rhl* and housekeeping gene expression across 50,000 cells. SD in gene expression was significantly influenced by the gene type (i.e. *las* vs. *rhl* vs. housekeeping genes; ANCOVA: $F_{2,234}$ = 23.48, p < 0.0001), time (quadratic term: $F_{1,234}$ = 25.60, p < 0.0001) and their interaction ($F_{2,234}$ = 0.71, p = 0.0045). SD was significantly higher for the *las* and *rhl* genes than for the housekeeping genes (TukeyHSD pairwise comparisons: *las* and *rhl* genes vs. housekeeping genes, both comparisons P_{adj} < 0.0001).Yellow dots connected with the dashed line represent the average standard deviation across the three housekeeping genes. Data are shown as the mean ± standard error of the standard deviation values across three independent replicates.



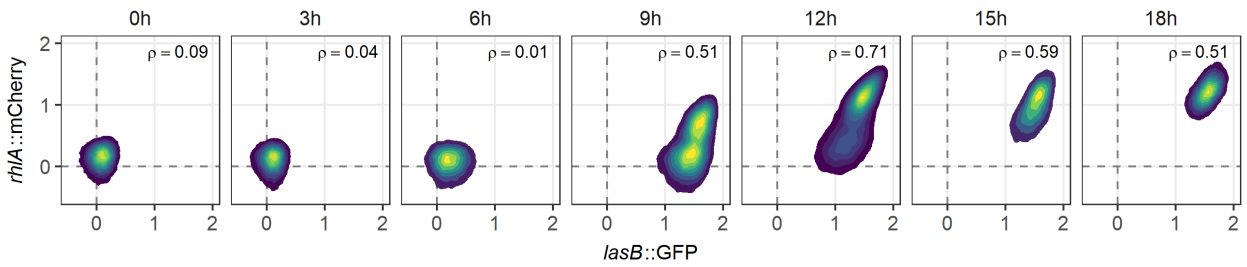
Supplementary Figure 5. Background fluorescence controls. Background fluorescence in the GFP and mCherry channels when measured using (a) a non-fluorescent strain carrying an integrated double reporter construct of promoterless *gfp* and *mCherry* strain (PAO1 WT::*empty-gfp-empty-mCherry*); (b) constitutively-expressing *gfp* but promoterless *mCherry* strain (PAO1 WT::*rpsL-gfp-empty-mCherry*), and (c) promoterless *gfp* but constitutively-expressing *mCherry* (PAO1 WT::*empty-gfp-rpsL-mCherry*). There was no evidence of cross fluorescence, demonstrating that the built in terminator sites ensure that the GFP and mCherry constructs are independent. Data across single cells is plotted as 2D density plot, where yellow represents the densest region and blue as the least dense region. Dotted lines represent mean background fluorescence in the mCherry and GFP channels.



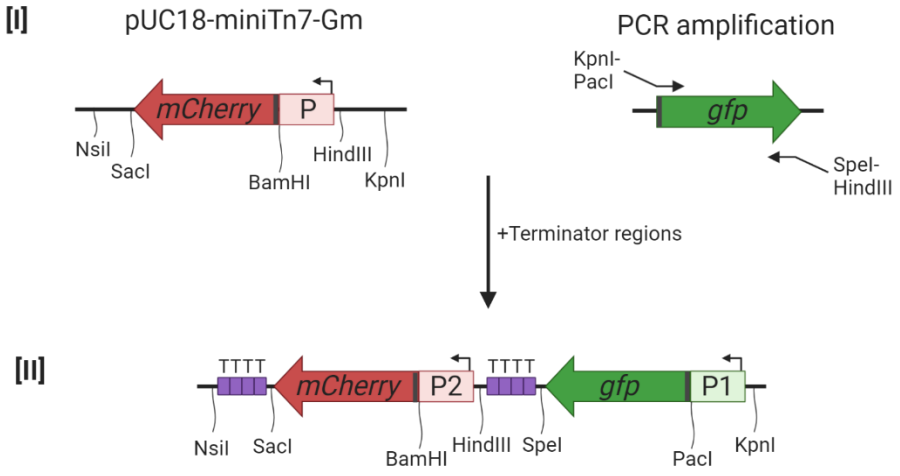
Supplementary Figure 6. Double reporter control experiments. Double gene expression reporters, where the promoter of the same gene – the housekeeping gene *rpsL*; and the QS gene *lasB* – were fused to both the GFP and mCherry. Panels show the averaged Spearman's rank correlation coefficient across three independent replicates (data of a single replicate is presented as a 2D density plot in Fig. 6a). Correlations are calculated using Spearman's rank correlation coefficient. Dotted lines represent zero correlation in gene expression. Data show the mean ± standard deviation of the correlation coefficient across three independent replicates.



Supplementary Figure 7. Swapped fluorescent gene reporter control. Correlation coefficient of QS gene expression when fluorescent gene reporters are swapped in the double reporter construct (black). Correlations are calculated using Spearman's rank correlation coefficient. Dotted lines represent zero correlation in gene expression. Data shows the mean ± standard deviation of correlation coefficient across three independent repeats.



Supplementary Figure 8. End level Las- and Rhl-QS commitment is positively correlated across cells. Simultaneous single-cell expression of Las- and Rhl-regulated traits (*lasB* and *rhlA*, respectively) was measured using double fluorescent gene reporters. Both genes were initially not expressed, but positive correlations built up over time as cells began to commit to QS. Fluorescence data across 50,000 single cells are shown as 2D density plot, where yellow and blue areas represent the densest and least dense regions, respectively. Dotted lines represent mean background fluorescence in the mCherry and GFP channels. Spearman's rank correlation coefficient (ρ) between the expression of two genes are shown in each panel. Data stems from one representative experiment out of a total of three independent replicates.



Supplementary Figure 9. Double fluorescent gene reporter scaffold. [I] Fluorescent gene marker *gfp* (*gfpmut3*) was PCR amplified (using primers denoted as arrows with unique restriction enzymes) and ligated to the pUC18-mini-Tn7-Gm-mCherry vector plasmid containing an empty promoter site fused to *mCherry*. [II] Promoter sites are denoted as P1 (fused to *gfp*) and P2 (fused to *mCherry*). Promoter regions of genes of interest were added at site P1 or P2 using restriction enzyme sites, KpnI and PacI, or, HindIII and BamHI, respectively. Each terminator region has four rho-independent terminators denoted as “T” (purple boxes). Ribosomal binding sites (RBSs) are shown as dark grey rectangles at the start of the fluorescent gene markers *gfp* and *mCherry*.

Supplementary Table 1. List of bacterial strains

Strains	Description	Source or reference
E. coli		
CC118 λpir	Δ (*ara, leu*)$_{7697}$ *araD*139 *ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB* (Rf^{R}) *argE(am) recA1 λpir*$^{+}$	[1]
P. aeruginosa		
PAO1 wild type	Wild type strain (ATCC 15692)	This laboratory
PAO1 *ΔlasR*	Deficient in the receptor of Las system	This laboratory
PAO1 *ΔrhlR*	Deficient in the receptor of Rhl system	This laboratory
PAO1 *ΔlasR ΔrhlR*	Deficient in the receptor of both Las and Rhl systems	This laboratory
***P. aeruginosa* wild type PAO1 (single fluorescent gene reporters)**		
promoterless-mCherry	Non-fluorescent strain with an empty promoter site fused to mCherry	This study
lasI-mCherry	Transcriptional fusion *lasI*-mCherry from pSR01	This study
lasR-mCherry	Transcriptional fusion *lasR*-mCherry from pSR02	[2]
lasB-mCherry	Transcriptional fusion *lasB*-mCherry from pSR03	This study
rhlI-mCherry	Transcriptional fusion *rhlI*-mCherry from pSR04	This study
rhlR-mCherry	Transcriptional fusion *rhlR*-mCherry from pSR05	[2]
rhlA-mCherry	Transcriptional fusion *rhlA*-mCherry from pSR06	This study
rpsL-mCherry	Transcriptional fusion *rpsL*-mCherry from pSR07	This study
rpoD-mCherry	Transcriptional fusion *rpoD*-mCherry from pSR08	This study
recA-mCherry	Transcriptional fusion *recA*-mCherry from pSR09	This study
***P. aeruginosa* wild type PAO1 (double fluorescent gene reporters)**		
empty-mCherry-empty-GFP	Non-fluorescent strain with an empty promoter site 1 fused to GFP, and empty promoter site 2 fused to mCherry	This study
lasI-GFP-lasR-mCherry	Transcriptional fusion *lasI-GFP* and *lasR-mCherry* from pDR01	This study
lasR-GFP-lasB-mCherry	Transcriptional fusion *lasR-GFP* and *lasB-mCherry* from pDR02	This study
rhlI-GFP-rhlR-mCherry	Transcriptional fusion *rhlI-GFP* and *rhlR-mCherry* from pDR03	This study
rhlR-GFP-rhlA-mCherry	Transcriptional fusion *rhlR-GFP* and *rhlA-mCherry* from pDR04	This study

lasR-GFP-rhlR-mCherry	Transcriptional fusion *lasR-GFP* and *rhlR-mCherry* from pDR05	This study
lasB-GFP-rhlA-mCherry	Transcriptional fusion *lasB-GFP* and *rhlA-mCherry* from pDR06	This study
rhlA-GFP-lasB-mCherry	Transcriptional fusion *rhlA-GFP* and *lasB-mCherry* from pDR07	This study
rpsL-GFP-rpsL-mCherry	Transcriptional fusion *rpsL- GFP* and *rpsL-mCherry* from pDR08	This study
lasB-GFP-lasB-mCherry	Transcriptional fusion *lasB- GFP* and *lasB-mCherry* from pDR09	This study
rpsL-GFP-empty-mCherry	Transcriptional fusion *rpsL-GFP* and *empty-mCherry* from pDR11	This study
empty-GFP-rpsL-mCherry	Transcriptional fusion *empty-GFP* and *rpsL-mCherry* from pDR12	This study

Supplementary Table 2. List of plasmids

Plasmid name	Description	Source or reference
pUX-BF13	Helper plasmid to provide Tn7 transposase proteins	[3]
pUC18-mini-Tn7-Gm	Gm^r on mini-Tn7; for chromosomal insertion in Gm^s bacteria in the *att*Tn7 site	[4]
pEX-A128-terminators	Commercial plasmid with terminator sequences between HindIII and SpeI sites.	[5]
pEX-A128-GFPmut3	Commercial plasmid with *gfp* sequence	This laboratory
Single fluorescent gene reporter plasmids		
pUC18-mini-Tn7-Gm-mCherry	Derived from pUC18-mini-Tn7-Gm; Gm^r on mini-Tn7; used for chromosomal insertion of *mCherry* at the *att*Tn7 site in Gm^s bacteria	This laboratory
pSR01	pUC18-mini-Tn7-Gm-mCherry with *lasI-mCherry*	This study
pSR02	pUC18-mini-Tn7-Gm-mCherry with *lasR-mCherry*	[2]
pSR03	pUC18-mini-Tn7-Gm-mCherry with *lasB-mCherry*	This study
pSR04	pUC18-mini-Tn7-Gm-mCherry with *rhlI-mCherry*	This study
pSR05	pUC18-mini-Tn7-Gm-mCherry with *rhlR-mCherry*	[2]
pSR06	pUC18-mini-Tn7-Gm-mCherry with *rhlA-mCherry*	This study
pSR07	pUC18-mini-Tn7-Gm-mCherry with *rpsL-mCherry*	This study
pSR08	pUC18-mini-Tn7-Gm-mCherry with *rpoD-mCherry*	This study
pSR09	pUC18-mini-Tn7-Gm-mCherry with *recA-mCherry*	This study
Double fluorescent gene reporter plasmids		
pUC18-mini-Tn7-Gm-mCherry-GFP	Derived from pUC18-mini-Tn7-Gm-mCherry; with amplified GFP from pEX-A128-GFP	This study
pDR01	pUC18-mini-Tn7-Gm with *lasI-GFP* and *lasR-mCherry*	This study
pDR02	pUC18-mini-Tn7-Gm with *lasR-GFP* and *lasB-mCherry*	This study

pDR03	pUC18-mini-Tn7-Gm with *rhlI-GFP* and *rhlR-mCherry*	This study
pDR04	pUC18-mini-Tn7-Gm with *rhlR-GFP* and *rhlA-mCherry*	This study
pDR05	pUC18-mini-Tn7-Gm with *lasR-GFP* and *rhlR-mCherry*	This study
pDR06	pUC18-mini-Tn7-Gm with *lasB-GFP* and *rhlA-mCherry*	This study
pDR07	pUC18-mini-Tn7-Gm with *rhlA-GFP* and *lasB-mCherry*	This study
pDR08	pUC18-mini-Tn7-Gm with *rpsL-GFP* and *rpsL-mCherry*	This study
pDR09	pUC18-mini-Tn7-Gm with *lasB-GFP* and *lasB-mCherry*	This study
pDR10	pUC18-mini-Tn7-Gm with *rpsL-GFP* and *lasI-mCherry*	This study
pDR11	pUC18-mini-Tn7-Gm with *rpsL-GFP* and *empty-mCherry*	This study
pDR12	pUC18-mini-Tn7-Gm with *empty-GFP* and *rpsL-mCherry*	This study

Supplementary Table 3. List of primers. Restriction enzyme sites are underlined in the sequences.

Primer	Sequence (5'-3')	Template DNA	Construct
For cloning promoter region upstream of mCherry			
lasI_Fwd_HindIII	CAGTAAGCTTGCCCGGAAGGCCATGTTTTG	PAO1 gDNA	pSR01
lasI_Rev_BamHI	CAGTGGATCCTCATGGGCCAGTGGTATCGAGAAT	PAO1 gDNA	pSR01
lasR_Fwd_HindIII	ATGACAAGCTTTGGAAAAGTGGCTATGTCGC	PAO1 gDNA	pDR01
lasR_ Rev_BamHI	CGTTCGGATCCTTAGGCGCTCCACTCCAATTTTC	PAO1 gDNA	pDR01
lasB_Fwd_HindIII	CAGTAAGCTTCGAATTCCGCGGCCAGGAAAGCGTGCAA	PAO1 gDNA	pSR03, pDR02, pDR07 and pDR09
lasB_Rev_BamHI	CAGTGGATCCTCAATCGAACAACAGGTCAAGCGTA	PAO1 gDNA	pSR03, pDR02, pDR07 and pDR09
rhlI_Fwd_HindIII	CAGTAAGCTTAGAACATCCAGAAGAAGTTCG	PAO1 gDNA	pSR04
rhlI_Rev_BamHI	CAGTGGATCCTCATACTTCCAGCGATTCAGAGAG	PAO1 gDNA	pSR04
rhlR_Fwd_HindIII	TAACGAAAGCTTCCTGCAGGGCGACTTCTAC	PAO1 gDNA	pDR03, pDR05
rhlR_ Rev_BamHI	TTGCTGGATCCTCACTGCATCTGGTATCGCTCC	PAO1 gDNA	pDR03, pDR05
rhlA_Fwd_HindIII	CAGTAAGCTTCCTGGGCAAGAGCACCTACG	PAO1 gDNA	pSR06, pDR04, pDR06
rhlA_Rev_BamHI	CAGTGGATCCTCATATGCAAACCGATACCAACAGA	PAO1 gDNA	pSR06, pDR04, pDR06
rpsL_Fwd_HindIII	CAGTAAGCTTGTACCGGTCTGGCTTACCAC	PAO1 gDNA	pSR07 and pDR08
rpsL_Rev_BamHI	CAGTGGATCCTCAGTGTGCCGAGTTCGGCTTTT	PAO1 gDNA	pSR07 and pDR08
rpoD_Fwd_HindIII	CAGTAAGCTTTCGTCCAGGAGAACCTTGAA	PAO1 gDNA	pSR08
rpoD_Rev_BamHI	CAGTGGATCCTCATGTCTCGAATACGTTGATCC	PAO1 gDNA	pSR08

recA_Fwd_HindIII	CAGTAAGCTTAGTGAGCGCTGC CAGTTC	PAO1 gDNA	pSR09
recA_Rev_BamHI	CAGTGGATCCTCAGAATTGGCG TTCGATCTGTC	PAO1 gDNA	pSR09
For cloning promoter region upstream of GFP			
lasI_Fwd_Kpn1	CAGTGGTACCGCCCGGAAGGCC ATGTTTTG	PAO1 gDNA	pDR01
lasI_Rev_Pac1	CAGTTTAATTAATCATGGGCCAG TGGTATCGAGAAT	PAO1 gDNA	pDR01
lasR_Fwd_Kpn1	ATGACGGTACCTGGAAAAGTGG CTATGTCGC	PAO1 gDNA	pDR02 and pDR05
lasR_Rev_Pac1	CGTTCTTAATTAATTAGGCGCTC CACTCCAATTTTC	PAO1 gDNA	pDR02 and pDR05
lasB_Fwd_Kpn1	CAGTGGTACCCGAATTCCGCGG CCAGGAAAGCGTGCAA	PAO1 gDNA	pDR06 and pDR09
lasB_Rev_Pac1	CAGTTTAATTAATCAATCGAACA ACAGGTCAAGCGTA	PAO1 gDNA	pDR06 and pDR09
rhlI_Fwd_Kpn1	CAGTGGTACCAGAACATCCAGA AGAAGTTCG	PAO1 gDNA	pDR03
rhlI_Rev_Pac1	CAGTTTAATTAATCATACTTCCA GCGATTCAGAGAG	PAO1 gDNA	pDR03
rhlR_Fwd_Kpn1	TAACGAGGTACCCCTGCAGGGC GACTTCTAC	PAO1 gDNA	pDR04
rhlR_Rev_Pac1	TTGCTTTAATTAATCACTGCATCT GGTATCGCTCC	PAO1 gDNA	pDR04
rhlA_Fwd_Kpn1	CAGTGGTACCCCTGGGCAAGAG CACCTACG	PAO1 gDNA	pDR07
rhlA_Rev_Pac1	CAGTTTAATTAATCATATGCAAA CCGATACCAACAGA	PAO1 gDNA	pDR07
rpsL_Fwd_Kpn1	CAGTGGTACCGTACCGGTCTGG CTTACCAC	PAO1 gDNA	pDR08
rpsL_Rev_Pac1	CAGTTTAATTAATCAGTGTGCCG AGTTCGGCTTTT	PAO1 gDNA	pDR08
Construction of double fluorescent gene reporters			
GFP_Fwd_ KpnI_PacI	CAGTGGTACCCCTACCTTAATTA A GCCGCTTTAAGAAGGAGGTA	pEX-A128-GFP	pUC18-mini-Tn7-Gm-mCherry-GFP
GFP_Rev_ HindIII_SpeI	CAGTAAGCTTGGGATGACTAGTT CATAACCCCGCTACTCATCATTT G	pEX-A128-GFP	pUC18-mini-Tn7-Gm-mCherry-GFP
Fwd_Term_Nsi1	CAGTATGCATTAATAATAAACGC AGAAAGGC	pEX-A128-terminators	pUC18-mini-Tn7-Gm-mCherry-GFP

Rev_Term_Sac1	CAGTGAGCTCTGCAGGTCGTCT CGGATC	pEX-A128-terminators	pUC18-mini-Tn7-Gm-mCherry-GFP
Colony PCR			
Tn7L_rev[a]	GGGTGTAGCGTCGTAAGCTAAT	*E. coli* colonies	Colony PCR to check insertion in *E. coli*
mCherry_rev2[b]	GGATATCCGCTGGGTGTTTA	*E. coli* colonies	Colony PCR to check insertion in *E. coli*
PTn7L	ATTAGCTTACGACGCTACACCC	*P. aeruginosa* colonies	Colony PCR to check insertion in *P. aeruginosa*
PglmS-up	CTGTGCGACTGCTGGAGCTGA	*P. aeruginosa* colonies	Colony PCR to check insertion in *P. aeruginosa*

[a]To confirm insertion of promoter region upstream of GFP in the mini-Tn7 vector, colony PCR was performed using Tn7L_rev primer, together with the corresponding “Rev_PacI” promoter-specific primer.

[b]To confirm insertion of promoter region upstream of mCherry in the mini-Tn7 vector, colony PCR was performed using mCherry_rev2 primer, together with the corresponding “Fwd_HindIII” promoter-specific primer.

Supplementary References

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