Supplementary information

Materials and Methods

Growth conditions

All bacterial strains (Table 1) were grown on Luria-Bertani (LB) broth containing 10g tryptone, 5g yeast extract and 10g NaCl in 1 liter of distilled water at 37°C (except for *Agrobacterium tumefaciens* A136/pCF218/pMV26, which was grown at 30°C, and for *Vibrio fischeri* MJ-1, which was grown at 30 °C in LBM (LB+2% NaCl) medium).

Effect of total volatiles produced by *Pseudomonas aeruginosa* on quorum sensing reporter strains.

For examination of the effect of *P. aeruginosa* volatiles on various quorum sensing **(**QS) response regulators, *P. aeruginosa* PAO1 was inoculated with different QS-reporter strains (Table S1) in two separate compartments of bi partite Petri dishes. Such a compartmental inoculation apparatus enabled only the exchange of volatiles between *P. aeruginosa* culture and the examined reporter strain. For assays evaluating possible antagonism/synergism of *P. aeruginosa's* volatiles towards QS response regulators, the reporter strains exposed to *P. aeruginosa's* volatiles were inoculated with their relevant Acyl homoserine lacton (AHL) signalling molecule (Cayman Chemical Company, Ann Arbor, MI, USA). In these experiments 1 µl of N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and N-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) was added at a concentration of 1 µM, and 1 µl of N-butanoyl-homoserine lactone (C4-HSL) and N-octanoyl-homoserine lactone (C8-HSL) at a concentration of 100µM. For assay examining agonistic activity of *P. aeruginosa* volatiles, both strains were incubated without any addition of exogenous AHL. Following over night incubation, the colonies of the reporter strain were scraped from the agar, re-suspended in phosphate buffer saline (PBS; 0.1 M pH=7.4; 10.9 g l^{-1} of Na_2HPO_4, 3.2 g l^{-1} of NaH_2PO_4 and 9 g l^{-1} of NaCl) and measured for luminescence in a 96 well plate using infinite-F200 plate reader (Tecan Trading AG, Switzerland). Relative luminescence was calculated as the luminescence divided by the optical

density. Relative green fluorescence produced by *B. cenocepacia* H111-I/pAS-C8 was measured with an excitation wavelength of 465 nm and an emission wavelength of 535 nm.

Volatile profiles analysis

500 µl of medium with either PAO1 or PAO1 Δ*lasR* in triplicates were diluted with 500 µl of DDW. 5 µl of 1ppm benzylacetone in MeOH were added as an internal standard, to a final concentration of 0.33 µM or 5 ppb. Stir Bar Sorptive Extraction was carried out using a 1x10 mm PDMS-coated Twister bar (Gerstel GmbH, Mülheim an der Ruhr, Germany), for 8 h. The Twisters were wiped and rinsed with DDW and were subjected to Thermal Desorption coupled to a Programmed-temperature vaporization (PTV) injector (TDU-CIS-4, Gerstel). Desorption was carried out under TDU splitless conditions with 40 ml min^{-1} He flow, and a temperature gradient of 60°C min^{-1} from 20°C to 170°C with a 5 mins hold. The PTV inlet was was fitted with a quartz wool liner (Gerstel) and kept under -20°C for the duration of the desorption process, after which a temperature gradient of 12°C sec^{-1} ensued, up to 250°C with a 10 min hold.

A 7890 Gas Chromatograph (GC) coupled to a 5375 Mass spectrometer (MS) (Agilent technologies, Santa Clara, CA), fitted with a Rxi-XLB 30x0.25x0.25 Column (Restek, Bellafonte, PA) were used to run the analyses. GC oven temperature gradient was 40°C for 3 mins then 15°C min^{-1} to 280°C for 5 mins. MS was operated in positive EI scan (40-400 amu) mode, 70 eV energy. Obtained chromatograms were analyzed with Chemstation software (Agilent) and mass spectra were compared to Wiley9/NIST08 combined mass spectral library (Wiley and Sons, Hoboken, NJ) and/or NIST11 (NIST, Gaitersburg, MD). 2-aminoacetophenone (2-AA) and benzylacetone identification was verified with commercials standards (Sigma) for spectra and retention times.

Integration was carried out in Chemstation using chemstation integrator. Areas under the curve (AUCs) were normalized to the AUC of the internal standard.

Effect of 2-aminoacetophenone on specific QS-reporter strains

2-AA was applied to various reporter strains in order to evaluate whether it could inhibit or activate different QS response regulators. The reporter strains were grown overnight at 30 °C in LB medium with an appropriate antibiotic and then washed and diluted 1:100 with fresh LB medium, obtaining a concentration of approximately 10^7 cells ml^{-1}. 100 µl of the cultures were added per well to a 96-wells plate (Corning Inc., NY, USA. Cat. number 356701) in four replicates. Assays for antagonistic/synergistic activity were prepared by the addition of 2-AA together with a specific AHL to the reporter strains cultures. Agonism assay was carried out by the addition of 2-AA to the reporter strain without the addition of any AHL. The negative controls lacked both 2-AA and AHL while the positive controls contained only the appropriate AHL at various concentrations. 2-AA was added for both agonism and antagonism/synergism assays at concentrations of 1, 10, 25, 50, 100 and 500 µM. C4-HSL, C8-HSL and 3-oxo-C12-HSL were added for positive controls at concentrations of 1, 10, 25, 50, 100 and 500 µM, while 3-oxo-C6-HSL was added at 1, 10, 25, 50 and 500 nM. For antagonism/synergism assays C4-HSL, C8-HSL and 3-oxo-C12-HSL were added at concentration of 10 µM, while 3-oxo-C6-HSL was added at 10 nM. 2-AA was added directly to the culture of the reporter strains before dividing it to the wells of the 96-well plate, while one microliter of various AHLs at different concentrations, dissolved in acetonitrile, was placed in the well half an hour prior to the addition of the cultures to allow evaporation of acetonitrile. The bacteria within the plates were then incubated for 24 h at 37 °C, except for *A. tumefaciens* A136/pCF218/pMV26, which was incubated at 30 °C. During the incubation, optical density (OD λ=600nm) and the luminescence or the fluorescence produced by the reporter strains were measured at 30 min intervals using infinite-F200 plate reader (Tecan Trading AG, Switzerland).

The effect of 2-AA in its volatile state was examined as follows: Briefly, 10 nmol of 2-AA and 100 µl of overnight incubated reporter strain were added to 2 opposite sides of bi partite Petri dishes.

Following over night incubation of the reporter strain was scraped from the agar plate and relative luminescence was measured as describe above.

Effect of 2-aminoacetophenone on *Vibrio fischeri's* LuxR-regulated luminescence.

2-Acetoaminophenone was added to *V. fischeri* in order to verify the activity of 2-AA on QS-regulated traits in a LuxR-harboring wild-type strain. The starters for the experiment were prepared as follow: prior each experiment, a culture from a glycerol stock was inoculated in LBM medium and incubated overnight at 30 ^{0}C, then diluted 1:1000 and incubated overnight again. The culture was then washed and diluted 1:1000 prior to addition of 25, 50 or 100 µM of 2-AA, or 10 nM of 3-oxo-C6-HSL. Luminescence and absorbance of MJ-1 cultures incubated in 96-well plate was measured as described above. It should be mentioned that in two repeats of the experiment, no luminescence was measured neither following the addition of AHL nor 2-AA. These repeats were not included in data analysis and presentation.

Effect of 2-AA analogs on LuxR

Seven analogs of 2-AA were tested against *E. coli*/pSB401 and *E. coli* JLD271/pAL103 in order to evaluate what chemical groups of 2-AA are involved in ligand-receptor interaction. The following compounds were used: 4-aminoacetophenone, 3-aminoacetophenone, aminoacetophenone, 2-nitroacetophenone, methyl anthranilate, anthranilic acid and 2-aminobenzaldehyde (Sigma, St. Louis, USA). These compounds were applied to the reporter strain in the concentrations of 1-50 µM as describe for 2-AA. Luminescence was measured after 12 h in a plate reader.

Multiple sequence alignment

Multiple sequence analysis (MSA) was done on TraR (PDB code: 1L3L), LasR (PDB code: 2UVO), SdiA (PDB code: 2AVX) and LuxR (Uniprot entry: P12746), using T-Coffee (http://tcoffee.vital-it.ch/apps/tcoffee/index.html). In addition, LuxR response regulators of the following species were aligned with LuxR of *Vibrio fischeri* (accession number CAA68561.1): *Aliivibrio logei* (AAQ90213.1), *Vibrio mimicus* (AAQ90214.1), *Photobacterium leiognathi*

(AAQ90227.1) and *Vibrio parahaemolyticus* (AAQ90194.1).

Homology Model Construction

LuxR (Uniprot entry: P12746) was aligned with TraR (PDB code: 1L3L) using T-Coffee (http://tcoffee.vital-it.ch/apps/tcoffee/index.html). A model of LuxR was created using the Modeller protocol (1) as implemented in Discovery Studio 4.0 (DS 4.0, Accelrys). Twenty models were generated and model quality was assessed using the protein report tool (DS 4.0) and the model with the best score was chosen for further refinement, which included minimization. Default protocol settings were used.

Identification of a binding site in the model

Binding site was defined using 'define binding site' protocol in DS 4.0. This protocol is based on an 'eraser and flood-filling grid algorithm', where binding sites are identified based on the shape of the receptor. The best scored site was determined as the binding site for the generated model. Default algorithm settings were used.

Ligand Docking

Ligands were prepared using ‘prepare ligands’ protocol and conformations were generated using ‘generate conformations’ protocol, both as implemented in DS 4.0. Docking of the ligands was performed using CDocker protocol (DS 4.0). Default protocols settings were used.

Supplementary figures and tables

Table S1. Bacterial strains and plasmids used in this study. C4-HSL: N-butanoyl-homoserine lactone; 3-oxo-C6-HSL: N-3-oxo-hexanoyl-homoserine lactone; C6-HSL: N-hexanoyl-homoserine lactone; 3-oxo-C8-HSL: N-3-oxo-octanoyl-homoserine lactone; C8-HSL**:** N-octanoyl-homoserine lactone; 3-oxo-C12-HSL: N-3-oxo-dodecanoyl-homoserine lactone.

Strain	**Genotype/relevant characteristics**	**source**
Vibrio fischeri MJ-1	Wild type	Prof. Ehud Banin laboratory collection.
P. aeruginosa PAO1	Wild type	
P. aeruginosa PAO1 Δ*lasR::*Tc^r	Mutant deficient in LasR response regulator. Mutation generated according to Rahim et al. [1]	Prof. Ehud Banin laboratory collection.
QS reporter strains		
Strain/plasmid (antibiotic resistance)	**Genotype/relevant characteristics**	**Source**
E. coli harboring pSB401(Tc^r)	Luminescent strain expressing LuxR of *Vibrio fischeri*, activated by C4-HSL, 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL and C8-HSL.	[2]
E. coli JLD271/pAL103 (Tc^r)	Luminescent strain expressing LuxR (as pSB401).	[3]
E. coli JLD271/pAL104 (Tc^r)	LuxR-negative strain.	[3]
E. coli/pSB536 (Amp^r)	Luminescent strain expressing AhyR of *Aeromonas hydrophila,* activated by C4-HSL.	[4]
P. aeruginosa JP2/pKD201(Tmp^r)	Luminescent strain expressing native LasR of *P. aeruginosa,* activated by 3-oxo-C12-HSL.	[5]
P. aeruginosa JP2/pKD-rhlA	Luminescent strain expressing cognate RhlR of *P. aeruginosa,* activated by C4-HSL.	[5]

(Tmp^r)		
Salmonella enterica 14028/pBA405E (Tc^r)	Luminescent strain expressing cognate SdiA response regulator, activated by 3-oxo-C6-HSL and 3-oxo-C8-HSL.	[6]
Agrobacterium tumefaciens A136/pCF218/pMV26 (Tc^r)	Luminescent strain expressing cognate TraR response regulator, sensitive to C8-HSL, C6-HSL and 3-oxo-C6-HSL.	[7]
Burkholderia cenocepacia H111-I/pAS-C8 (Gm^r)	Fluorescent strain expressing its CepR response regulator, activated by C8-HSL.	[8]

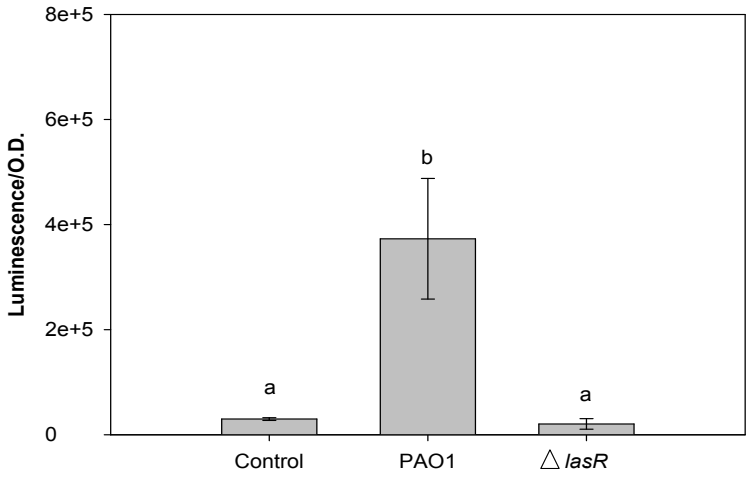


Figure S1. Effect of total volatiles of *P. aeruginosa* wild type and Δ*lasR* mutant on LuxR-expressing biosensor. Luminescence levels of *Escherichia coli*/pSB401 reporter strain, expressing LuxR response regulator in response to total volatiles of *P. aeruginosa* wild type (PAO1) and its *lasR* mutant (Δ*lasR*). n=4; Error bars are the standard error of the mean. Different letters indicate a statistical difference (P<0.05) according to ANOVA on Ranks and Student–Newman–Keuls post hoc test.

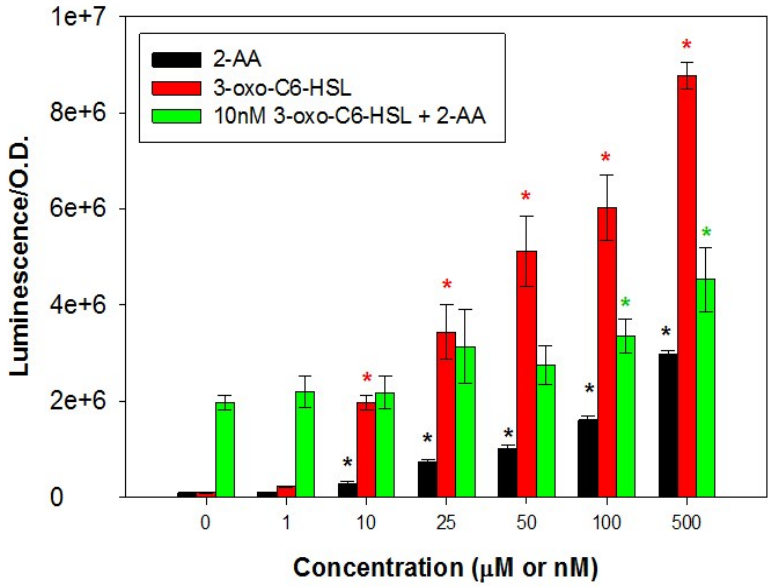


Figure S2. The effect of 2-AA and 3-oxo-C6-HSL on LuxR-expressing biosensor. Relative luminescence of the LuxR-expressing *E. coli* JLD271/pAL103 reporter strain in the presence of 0-500 µM 2-AA and 0-500 nM 3-oxo-C6-HSL. For antagonistic/synergistic assays (3-oxo-C6-HSL + 2AA), 0–500 nM of 2-AA was added to the reporter strain in the presence of 10 nM of 3-oxo-C6-HSL. Presented values are the luminescence measurements taken 12 h post exposure to signalling molecules. n = 4; error bars represent standard error of the mean, asterisks indicate a statistical difference ($P < 0.01$) compared to control, according to ANOVA and the Dunnett post hoc test.

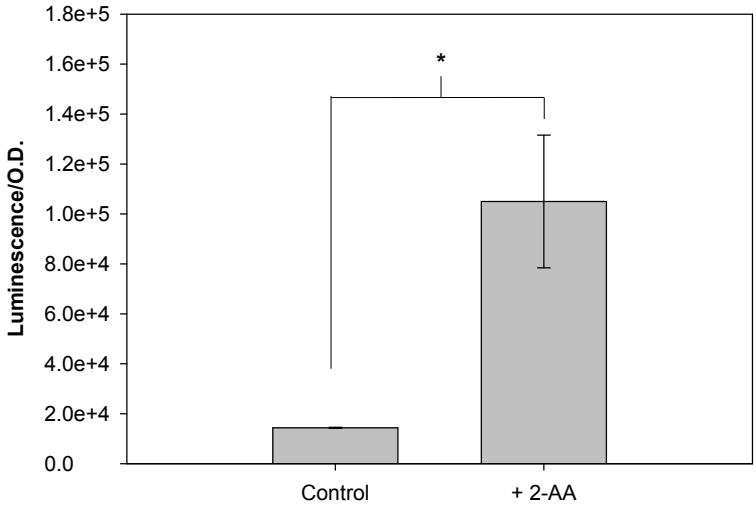


Figure S3. The effect of 2-aminoacetphenone, applied as a volatile, on LuxR-expressing biosensor. Relative luminescence of *E. coli*/pSB401 measured after overnight exposure to 10 nmol of 2-aminoacetophenone (2-AA) added to the opposite compartment of a bipartite petri dish. n=4; Error bars represent standard error of the mean, asterisk represents significant difference between the groups according to t-test ($P<0.05$).

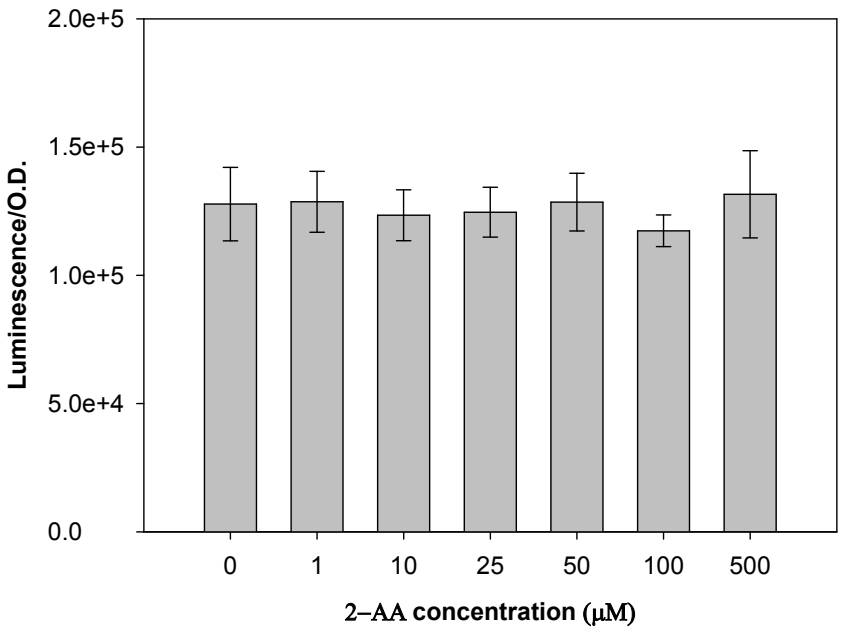


Figure S4. The effect of 2-AA on LuxR-negative biosensor. Relative luminescence of *E. coli* JLD271/pAL104 strain in the presence of 0-500 μM of 2-AA. Measurements were carried out at 12 h post exposure to signalling molecule. n=4; Error bars represent standard error of the mean. No statistical differences were observed (P>0.05) according to ANOVA.

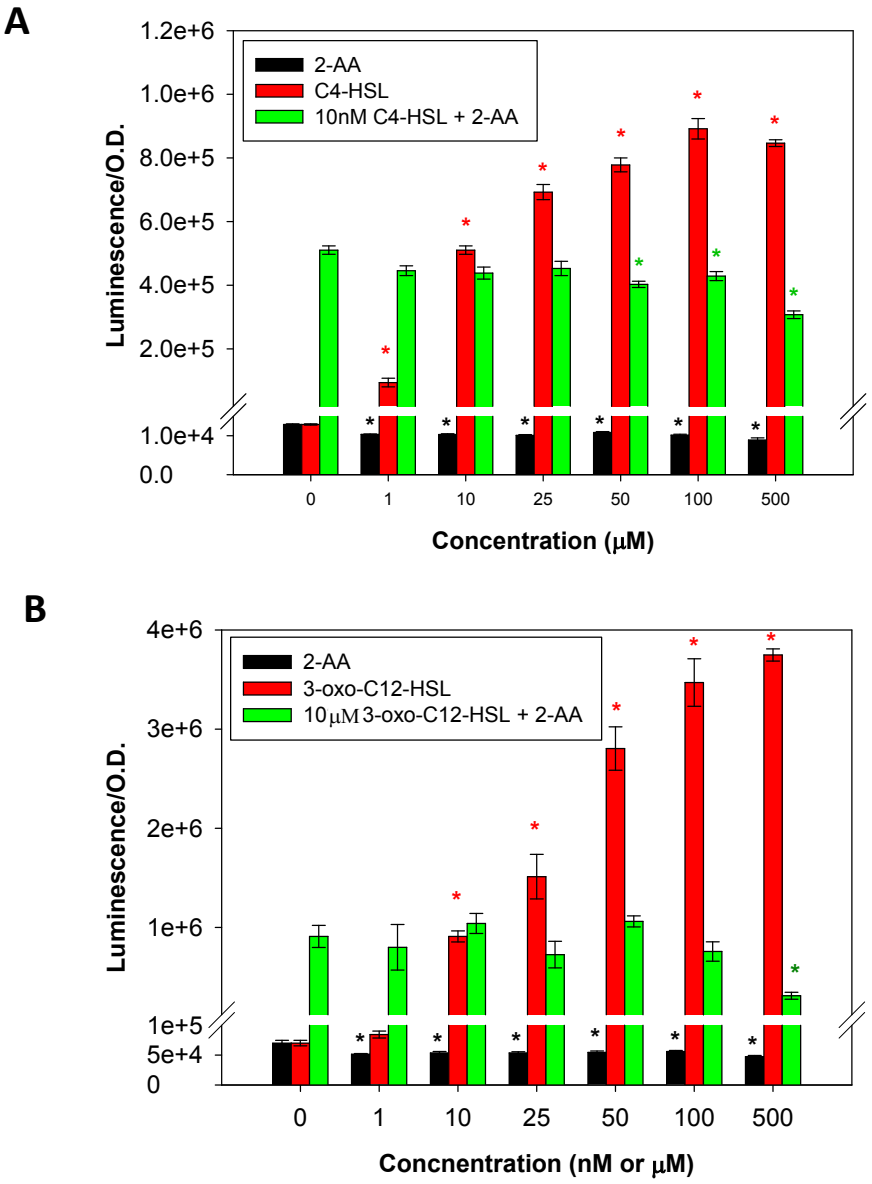
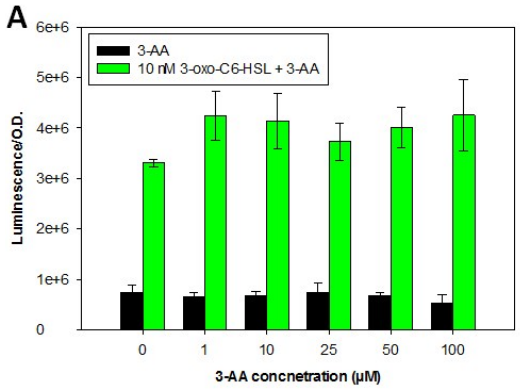
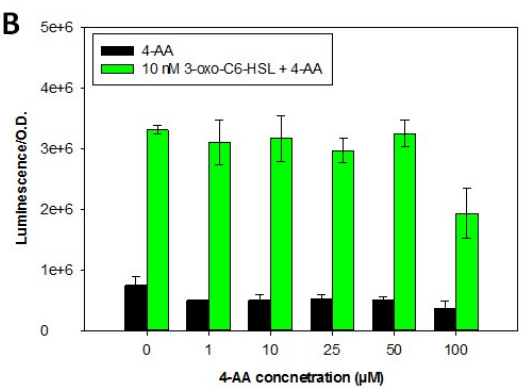
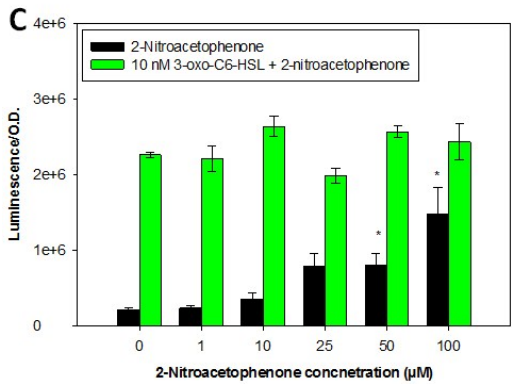
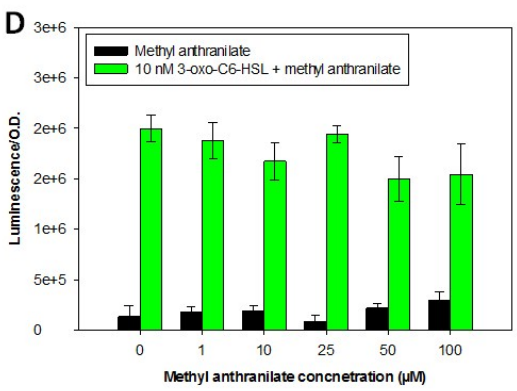


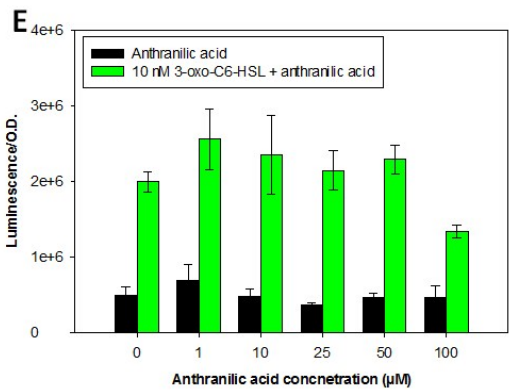
Figure S5. The effect of 2-AA on RhlR- and LasR-expressing biosensors. Relative luminescence of reporter strains harboring *P. aeruginosa* cognate QS receptors, RhlA (*P. aeruginosa* JP2/pKD-rhlA, panel A) and LasR (*P. aeruginosa* JP2/pKD201 panel B), exposed to 2-AA and the relevant Acyl homoserine lacton (AHL). 2-AA and C4-HSL were added at 0-500 μM and 3-oxo-C12-HSL was added at 0-500 nM. For antagonistic/synergistic assays (C4-HSL +2AA and 3-oxo-C12-HSL + 2-AA), 2-AA was added to the reporter strain in the presence of 10 nM (panel A) or 10 μM (panel B) of the cognate AHL. n=4; Error bars represent standard error, asterisks indicate statistical difference (P<0.01) compared to control, according to ANOVA and Dunnett post hoc test.

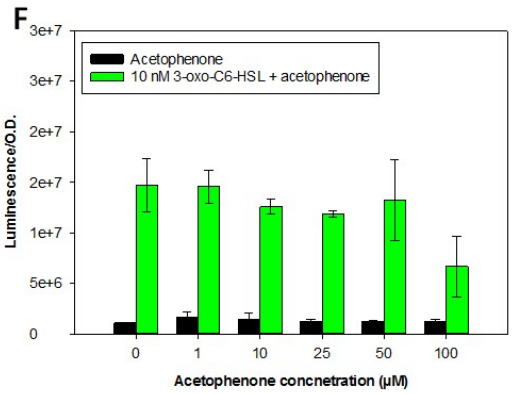

A
3-AA
10 nM 3-oxo-C6-HSL + 3-AA
Luminescence/O.D.
3-AA concnetration (μM)


B
4-AA
10 nM 3-oxo-C6-HSL + 4-AA
Luminescence/O.D.
4-AA concnetration (μM)


C
2-Nitroacetophenone
10 nM 3-oxo-C6-HSL + 2-nitroacetophenone
Luminescence/O.D.
2-Nitroacetophenone concnetration (μM)


D
Methyl anthranilate
10 nM 3-oxo-C6-HSL + methyl anthranilate
Luminescence/O.D.
Methyl anthranilate concnetration (μM)


E
Anthranilic acid
10 nM 3-oxo-C6-HSL + anthranilic acid
Luminescence/O.D.
Anthranilic acid concnetration (μM)


F
Acetophenone
10 nM 3-oxo-C6-HSL + acetophenone
Luminescence/O.D.
Acetophenone concnetration (μM)

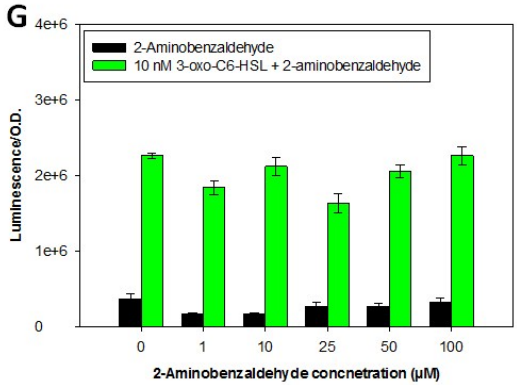

G
2-Aminobenzaldehyde
10 nM 3-oxo-C6-HSL + 2-aminobenzaldehyde
Luminescence/O.D.
2-Aminobenzaldehyde concnetration (μM)

Figure S6. The effect of chemical analogues of 2-AA on LuxR-expressing biosensor. Relative luminescence of *E. coli* JLD271/pAL103 reporter strain upon exposure to 1 - 50µM of the following substances: 3-aminoacetophenone (3-AA), 4-aminoacetophenone (4-AA), 2-nitroacetophenone, methyl anthranilate, anthranilic acid, acetophenone and 2-aminobenzaldehyde. The compound were tested for inducing activity in absence of AHL (black bars) and for synergistic/antagonistic activities in presence of AHL (green bars). Measurements are after 12h of incubation. n=4; Error bar represent standard error of the mean. Asteriks indicate a statistical difference relative to control (0 µM) (P<0.05) according to ANOVA and Dunnet post hoc test versus control.

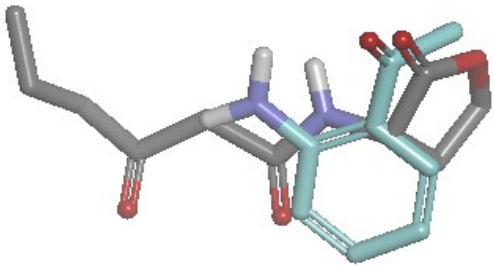


Figure S7. Overlap of the docked 3-oxo-C6-HSL and 2-AA, within the binding pocket of LuxR. To facilitate visualization, amino acids interacting with the compounds are not shown. For relevant residues of LuxR, please see Figure 5.

```
LuxR     1 MKNINADD-TY-----RIINKIKACRA-YDINQCLSDMTKMVHCEYYLTLAIIYPHS
LasR     1 GAMALVDG--F-----LELE--RSSGK-LEWSAILQKMASDLGFSKI-LFGLLPKDS
TraR     1 MQHW-LDK--L-----TDLA--AIEGDECILKTGLADIADHFGF-----TGYAYLH-
SdiA     1 MSDK--DFFSWRRTMLLRFQ--RMETA-EEVYHEIELQAQQLEYDYY-SLCVRHPVP

LuxR    51 MVKSDISILDNYPKKWRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE-NNAVNKKS
LasR    47 QDYENAFIVGNYPAAWREHYDRAGYARVDPTVSHCTQSVLPIFWEPSI---YQTRKQ
TraR    42 IQHRHITAVTNYHRQWQSTYFDKKFEALDPVVKRARSRKHIFTWSGEHERPTLSKDE
SdiA    52 FTRPKVAFYTNYPEAWVSYYQAKNFLAIDPVLNPENFSQGHLMWNDD-----LFSEA

LuxR   107 PNVIKEAKTSGLITGFSFPIHTANNGFGMLSFAHSE-KDNYIDSLFLHA---CMNI
LasR   101 HEFFEEASAAGLVYGLTMPLHGARGELGALSLSVEA-ENRAEANRFMESVLPTLWM
TraR    99 RAFYDHASDFGIRSGITIPIKTANGFMSMFTMAS-D-KPVIDLDREIDAVAAAATI
SdiA   104 QPLWEAARAHGLRRGVTQYLMLPERALGFLSFSRCSAREIPILSDEL---QLKMQL

LuxR   159 PLIVPSLVDNYRKINIANNKS 179
LasR   156 -LKDYALQSGAGLAFEHPVSK 175
TraR   153 -GQIHARISF-LRTTPTAEDA 171
SdiA   157 -LVRES-----LMALMRLNDE 171
```

Figure S8. Multiple Sequence Alignment of the partial amino acid sequence of TraR, LasR, SdiA and LuxR (T-Coffee server). Conserved residues in binding sites are highlighted in yellow. Similar residues marked in blue, identical residues are marked in red. Residues interacting with 2-AA are marked by a black square.

```
Vibrio Fischeri           1 MKNINADDTYRIINKIKACRA-YDINQCLSDMT
Aliivibrio logei            MKDINADDTYRIINKIKACRSNNDINQCLSDMT
Vibrio mimicus              MKDINADDTYRIINKIKACRSNNDINQCLSDMT
Photobacterium leiognathi   MKDINADDTYRIINKIKACRSNNDINQCLSDMT
Vibrio parahaemolyticus     MKDINADDTYRIINKIKRCRSNKDINQCLSDMT
                            **:************** **:  **********

Vibrio fischeri          33 KMVHCEYYLTLAIIYPHSMVKSDISILDNYPKK
Aliivibrio logei            KMVHCEYYL-LAIIYPHSMVKSDISILDNYPKK
Vibrio mimicus              KMVHCEYYL-LAIIYPHSMVKSDISILDNYPKK
Photobacterium leiognathi   KMVHCEYYL-LAIIYPHSMVKSDISILDNYPKK
Vibrio parahaemolyticus     KMVHCEYYL-LAIIYPHCMVKSDISIVDNYPKK
                            ********* *******.********:******

Vibrio fischeri          66 WRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE
Aliivibrio logei            WRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE
Vibrio mimicus              WRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE
Photobacterium leiognathi   WRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE
Vibrio parahaemolyticus     WRQYYDDANLIKYDPIVDYSNSNHSPINWNIFE
                            ********************************

Vibrio fischeri          99 NNAVNKKSPNVIKEAKTSGLITGFSFPIHTANN
Aliivibrio logei            NNAVNKKSPNVIKEAKTSGLITGFSFPIHTANN
Vibrio mimicus              NNAVNKKSPNVIKEAKTSGLITGFSFPIHTANN
Photobacterium leiognathi   NNAVNKKSPNVIKEAKTSGLITGFSFPIHTANN
Vibrio parahaemolyticus     NNAVNKKSPNVIKEAKSSGLITGFSFPIHTANN
                            ****************:****************

Vibrio fischeri         132 GFGMLSFAHSEKDNYIDSLFLHACMNI
Aliivibrio logei            GFGMLSFAHSEKDNYIDSLFLHACMNI
Vibrio mimicus              GFGMLSFAHSEKDNYIDSLFLHACMNI
Photobacterium leiognathi   GFGMLSFAHSEKDNYIDSLFLHACMNI
Vibrio parahaemolyticus     GFGMLSFAHSEKDNYIDSLFLQACMNI
                            ********************:*****
```

Figure S9. Multiple Sequence Alignment of the LuxR response regulators of *Vibrio fischeri*, *Aliivibrio logei*, *Vibrio mimicus*, *Photobacterium leiognathi* and *Vibrio parahaemolyticus* (T-Coffee server). Residues that were shown to interact with 2-AA are marked in red.

References

1 R. Rahim, U. A. Ochsner, C. Olvera, M. Graninger, P. Messner, J. S. Lam, and G. Soberón-Chávez, *Mol. Microbiol*, 2001, **40**, 708–718.

2 M. K. Winson, S. Swift, L. Fish, J. P. Throup, F. Jørgensen, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. Stewart, *FEMS Microbiol. Lett.*, 1998, **163**, 185–192.

3 A. Lindsay and B. M. Ahmer, *J. Bacteriol*., 2005, **187**, 5054–5058.

4 S. Swift, A. V. Karlyshev, L. Fish, E. L. Durant, M. K. Winson, S. R. Chhabra, P. Williams, S. Macintyre, and G. Stewart, *J. Bacteriol*., 1997, **179**, 5271–5281.

5 K. Duan and M. G. Surette, *J. Bacteriol*., 2007, **189**, 4827–4836.

6 B. Michael, J. N. Smith, S. Swift, F. Heffron, and B. M. Ahmer, *J. Bacteriol*., 2001, **183**, 5733–5742.

7 P. Sokol, U. Sajjan, M. Visser, S. Gingues, J. Forstner, and C. Kooi, *Microbiol.*, 2003, **149**, 3649–3658.

8 K. Riedel, M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Høiby, M. Givskov, S. Molin, and L. Eberl, *Microbiol.*, 2001, **147**, 3249–3262.