1 **Directed evolution of *Vibrio fischeri* LuxR signal sensitivity**

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1 **Abstract**

2 LuxR is the core component of *Vibrio fischeri* quorum sensing. It acts as the
3 transcriptional activator by binding to its cognate signaling molecules 3-oxo-
4 hexanoyl-homoserine lactone (3OC6HSL). Although several acyl-HSLs with 3-oxo-
5 groups are known to activate LuxR with similar efficiency, acyl-HSLs without 3-oxo
6 groups are very weak inducers. We conducted a round of LuxR directed evolution to
7 acquire LuxR mutants with higher signal sensitivity to octanoyl-homoserine lactone
8 (C8HSL). All of the isolated mutants showed increased signal sensitivity to many
9 acyl-HSLs, including C8HSL, and some to the LuxR antagonist *p*-coumaroyl-HSL.
10 The evolution of ligand sensitivity proceeded through the stabilization of the signal-
11 bound state, thereby elevating the effective concentration of LuxR at the ON-state.

1 Introduction

2 Quorum sensing of Gram-negative bacteria is mainly mediated by acyl-homoserine
3 lactones (acyl-HSLs), which serve as signaling molecules (1). Diverse structural variation is
4 observed in acyl-HSLs that are biosynthesized in nature, and each acyl-HSL is synthesized
5 with the aim of activating/deactivating a cognate sensory protein. To clarify how this
6 remarkable diversity has arisen and to provide bacterial sensors (2) and cell-cell
7 communication devices (3, 4, 5, 6, 7) to the synthetic biology community, quorum sensing
8 proteins have been extensively targeted for mutagenesis (4, 8, 9).

9 The *Vibrio fischeri* LuxI/LuxR system is one of the most well-studied quorum sensing
10 systems among Gram-negative bacteria. In this system, 3-oxohexanoyl-HSL (3OC6HSL) is
11 synthesized by the enzyme LuxI, transported across the bacterial cell wall, and then
12 recognized by the sensor protein LuxR in both its source and other bacteria. Upon binding
13 to acyl-HSLs, LuxR undergoes a structural transition into the active dimer form and binds to
14 a specific DNA sequence called the lux box (10), thereby mediating the expression of the
15 downstream genes.

16 The functional plasticity (or evolvability) of LuxR has been explored extensively. Based on
17 structural modeling using the X-ray structure of TraR from *Agrobacterium tumefaciens* (11,
18 12), several residues have been shown to strongly influence the signal specificity of LuxR
19 (13). Arnold and her colleagues performed a directed evolution of LuxR (4, 8, 9) and

different sets of amino acid substitutions that alter its signal specificity and sensitivity. For instance, HSLs without 3-oxo groups are weak ligands for LuxR and thus require a high concentration (~10 μ M). Two rounds of mutagenesis followed by a screening search for mutants expressing higher fluorescent marker levels (green fluorescent protein, GFP) revealed that amino acid substitutions at positions 33, 45, 95, 116, 135 and 174, especially combination, conferred elevated sensitivity to octanoyl-HSL (C8HSL) (4, 8, 9).

In this work, we conducted a very similar directed evolution experiment of LuxR to achieve higher sensitivity to C8HSL. For this purpose, we adopted a new selection system, which we originally developed for the directed evolution of genetic circuits (14). In this method, the entire selection process occurs in liquid, thus allowing many sets of different selection programs to be conducted simultaneously. Using this method, we isolated C8HSL-sensitive LuxR mutants. An analysis of the isolated variants revealed that they were sensitized to all of the tested HSLs (C6HSL, C8HSL, 3OC6HSL, and 3OC8HSL), which suggests that there was an improvement in ligand sensitivity that proceeded through the stabilization of the signal-bound state, thereby elevating the effective concentration of the effectors in the ON-state. This “quick-and-dirty” solution may be the prevailing source of novel LuxR family protein functions, possibly due to the rapid evolution of quorum-sensing systems both in nature and in laboratories.

Materials and Methods

Bacterial strains, media, and growth conditions

E. coli strains DH10B (F^- *mcrA* $\Delta(mrr-hsdRMS-mcrBC)\Phi80d$ *lacZ* Δ M15 Δ *lacX74recA1* *endA1* *araD139* $\Delta(ara, leu)$ 7697 *galU galK* λ^- *rpsL* (Str^R) *nupG*) (Invitrogen, CA) and XL10 Gold (Tet^R $\Delta(mcrA)$ 183 $\Delta(mcrCB-hsdSMR-mrr)$ 173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* Hte [*F'**proAB* *lacIq* Δ M15 Tn10 (Tet^R) Tn5 Kan^R Amy]) (Stratagene, CA) were used for cloning and library construction. Δ *tdk* strain JW1226 (F^- $\Delta(araD-araB)$ 567 Δ *lacZ*4787($::rrnB$ -3) λ^- Δ *tdk*-747::*kan* *rph-1* $\Delta(rhaD-rhaB)$ 568, *hsdR*514) from the KEIO collection (15), harboring pAC(*Plux*)-*gfp*_{UV} or pAC(*Plux*)-HSV*tk*, was used for genetic selection. *E. coli* strains were incubated in LB medium (2.0% (w/v) Lennox LB; Invitrogen) or on LB agar plates (2.0% (w/v) LB, 1.5% (w/v) agar; Nacalai tesque, JP) at 37°C. Antibiotics were added at the following concentrations: 30 μ g ml⁻¹ chloramphenicol (Nacalai tesque) to maintain the pACYC-based vector and/or 50 μ g ml⁻¹ carbenicillin (Invitrogen) to maintain the pTrc-based vector.

The acyl-HSLs used in this study were 3OC6HSL, 3-oxooctanoyl-HSL (3OC8HSL), hexanoyl-HSL (C6HSL), C8HSL, and *p*-Coumaroyl-HSL (*p*CHSL). All of these compounds were purchased from Sigma-Aldrich (MO). These stock solutions (1-10 mM) were prepared by dissolving appropriate amounts of the compounds in ethyl acetate (Nacalai tesque) acidified with glacial acetic acid (0.01% (v/v); Nacalai tesque) and were stored at -20°C.

*pCH*SL stock solutions were prepared with ethanol instead of ethyl acetate (1-10 mM). For the liquid-phase experiments, we dispensed acyl-HSL stock solutions into autoclaved 96-deep-well plates, evaporated the ethyl acetate at room temperature, and then added the medium containing cells to the dried acyl-HSL.

Plasmid construction

The *luxR* reading frame was PCR-cloned into the *NcoI/HindIII* site of pTrc99A, resulting in pTrc-*luxR*. Similarly, the reading frames of *gfp_{UV}* from pGFP_{UV} (Clontech, CA) and herpes simplex virus thymidine kinase (HSV*tk*) (14) were PCR-cloned into the *NcoI/HindIII* site of pACYC184 (p15A-ori, Chloramphenicol resistance (Cm^R). The resultant pAC-*gfp_{UV}* and pAC-HSV*tk* were then PCR-cloned into *Clal/HindIII*, yielding pAC(*P_{trc}*)-*gfp_{UV}* and pAC(*P_{trc}*)-HSV*tk*, respectively. Next, the *trc* promoters of these plasmids were replaced with the *lux* promoter (5'-ACCTGTAGGATCGTACAGGTTTACGCAAGAAAATGGTTTGTATAGTCGAATAAA-3'), resulting in pAC(*P_{lux}*)-*gfp_{UV}* and pAC(*P_{lux}*)-HSV*tk*. The sequences of all of the above plasmids were confirmed using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, CA) and an ABI Prism 3100 Genetic Analyzer (Life Technologies Corporation).

Library construction

Whole-gene mutagenesis was performed using pTrc-*luxR* (1 ng) as a template and a 1 μ M concentration of each primer (primer 1: 5'-CAATCTGTGTGGGCACTCGAC-3' and primer 2: 5'-TACTGCCGCCAGGCAAATTC-3'). The reaction mixtures (50 μ l total volume) contained 5 U of Taq DNA polymerase (New England Biolabs, MA), 200 μ M of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, and 10 μ M $MnCl_2$, as described previously (16). The amplification factor was approximately 1,000 (ca. 10 effective rounds). The PCR product was subcloned into the *NcoI/HindIII* site of pTrc99A. The ligation mixture was transformed into Electro MAX DH10B by electroporation. DNA from the transformant was isolated by miniprep to yield the library plasmid pTrc-[*luxR*] (library size $\approx 10^6$).

Selection and Screening Process

The *luxR* library was introduced into JW1226 harboring pAC(*Plux*)-HSV*tk*, and the cells cultured overnight in 10 ml of LB medium containing the appropriate type and concentration of acyl-HSLs (in this case, 100 nM of C8HSL). The transformants ($\approx 10^6$ cells) from the were inoculated into ON-selection medium (2.0% (w/v) tryptone; Becton, Dickinson and Company, MD, 0.5%(w/v) NaCl; Nacalai tesque, 10 μ g ml⁻¹ thymidine; Wako, JP, 1 μ g ml⁻¹ adenosine; Tokyo Chemical Industry Co., LTD., JP, and 20 μ g ml⁻¹ 2'-deoxy-5-fluorouridine (5FdU); Sigma-Aldrich) and incubated for 20 h. 5FdU is a potent inhibitor of thymidine

synthase, in the presence of which cells fail to biosynthesize dTMP, a precursor of genomic DNA. In this situation, only those cells expressing HSVTK can grow due to the capability of HSVTK to phosphorylate exogenously added thymidine. From the pool of these “ON-cells, plasmid DNA was isolated.

Site-directed mutagenesis

Using the ExSite method (Stratagene), PCR-based site-saturation or substitution mutagenesis was performed on the pTrc-*luxR* template (10-20 ng) using an appropriate primer set with altered codon sequences for the targeted site. The PCR fragments were subcloned into the vector *by* using self-ligation. The ligation products were transformed into XL10 Gold cells, plated and grown overnight on LB agar plates to isolate clones.

Similarly, site-saturation mutagenesis was induced for cysteine at amino acid position 245 (C245) using ExSite PCR with the primers containing NNK sequence (N = equimolar mixture of dATP, dCTP, dGTP, and dTTP; K = equimolar mixture of dGTP and dTTP) at the targeted sites. The resultant plasmids were transformed into DH10B. The transformants were plated on LB agar plates. Approximately 10^3 colonies from each plate were pooled, and their plasmid DNA was isolated for subsequent analysis.

GFP-reporter analysis of gene expression

For rapid screening, the library plasmids were transformed into JW1226 containing pAC(*Plux*)-*gfp_{UV}* and incubated on LB agar plates overnight. These colonies were picked and inoculated into 500 μ L of LB medium in 96-well deep-well plates. The cultures were spotted with a 96-well pin replicator onto LB agar plates containing acyl-HSL. After 12-20 h, LuxR mutants with the desired phenotype were visually selected under 365 nm UV light.

For the quantitative assay, JW1226 harboring pAC(*Plux*)-*gfp_{UV}* and plasmids encoding variant LuxR genes were first grown overnight from single colonies or glycerol stocks in LB medium, and then the cultures were diluted 1,000-fold into 500 μ L of fresh LB medium containing appropriate antibiotics and acyl-HSLs in 96-well deep-well plates (Qiagen, CA). These cultures were shaken for 12 h. The cultures (20 μ L each) were diluted 10-fold into fresh LB medium (a total volume of 200 μ L) in 96-micro-well plates (Nacalai tesque). GFP_{UV} fluorescence (390 nm excitation, 510 nm emission) was measured using a fluorescence microplate reader (Thermo Fisher Scientific Inc., FLUOROSKAN ASCENT); cell densities were measured using a microplate reader (Molecular Devices, SpectraMax Plus 384) at 600 nm. The fluorescence of the cell suspensions was normalized to their optical densities.

Western blot analysis of LuxR variants

A histidine hexamer was fused *in frame* to the N-terminus of the reading frames of LuxR_{WT} and its variants (LuxR_{C8-1}, LuxR_{C8-2} and LuxR_{N86K-C245W}). To perform this manipulation, we

1 inserted 5'-CATCATCATCATCAT-3' immediately downstream of the start codon (ATG)
2 the *luxR* gene using ExSite PCR.

3 The resultant plasmids were introduced into JW1226 (*Plux-gfp_{UV}*). The transformants
4 were incubated in 2 ml of LB medium overnight. A 300- μ L aliquot of the culture was added to
5 40 ml of fresh LB medium with or without 1 μ M 3OC6HSL and shaken at 37°C for 3 h. The
6 cells were subsequently centrifuged at 3,300 rcf for 5 min to collect the resultant cell pellet.
7 The cells were resuspended to a concentration of 1.2×10^{10} cells/mL in sonication lysis
8 buffer (20 mM HEPES, 500 mM NaCl, 25 mM imidazole, 5% (v/v) glycerol, 0.1 mg/mL
9 lysozyme, and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics,
10 Mannheim, Germany), pH 7).

11 After chilling on ice for 30 mins, the cell suspension was sonicated on ice, from which we
12 collected 300 μ L as the lysate. The remainder of the lysate was rigorously centrifuged ($1.7 \times$
13 10^4 rcf for 30 min), and the supernatant was taken as soluble fraction. The total and soluble
14 fraction thus obtained was diluted with 2 $\times$ SDS sample buffer (0.15 M Tris, 1.2% (w/v) SDS,
15 30% (v/v) glycerol, 15% (v/v) mercaptoethanol, and 0.18 mg/L bromophenol blue), and
16 boiled for 5 min. From each sample, 15 μ L (9×10^7 cells) was loaded on an acrylamide gel
17 (12% (w/v)).

18 The protein in the gel was then transferred to a PVDF membrane for 1 h at 100 mA and
19 washed with TBS-T solution (20 mM Tris-HCL, 0.8% NaCl, and 0.2% Tween 20). The

1 membrane was incubated for 90 min in 1 mL of TBS-T containing 5% skim milk with HRP-
2 HRP-conjugated anti-His-tag antibody (Clontech) at room temperature. The membrane was
3 then washed 3 times in TBS-T and incubated in DAB (Nacalai tesque) solution for 15 min.
4 Finally, the membrane was washed with deionized water for image analysis.
5

Results

LuxR-Plux system for acyl-HSL detection

We adopted a two-plasmid system to evaluate and screen for the function of LuxR and its mutants (**Fig. 1A**). One plasmid, pTrc-*luxR* constitutively expressed *luxR* under the *trc* promoter (17), and the leaky expression of this promoter was sufficient to support all cellular functions of LuxR. The second plasmid, pAC-*Plux-gfp_{UV}* encoded *gfp_{UV}* under the control of the *lux* promoter. Co-transformation with these two plasmids allowed *E. coli* cells to fluoresce in response to acyl-HSLs. We found that wild-type LuxR (LuxR_{WT}) required 20-25 nM of its cognate signaling molecule 3OC6HSL to achieve a half-maximal level of gene activation (EC₅₀) (**Fig. 2A**). LuxR_{WT} was also activated by acyl-HSLs lacking a 3-oxo group (C6HSL and C8HSL), but this only occurred in the presence of high concentrations of these compounds (EC₅₀ > 1 μ M). These results were in good agreement with previous reports (18, 19).

Directed evolution of LuxR for increased sensitivity to C8HSL

To isolate LuxR mutants with elevated sensitivity to C8HSLs, we conducted directed evolution (**Fig. 1B**) using the selection system that we recently developed for the selection of genetic circuits (14).

We conducted error-prone PCR mutagenesis (16) on the whole reading frame of LuxR. The resultant PCR product was ligated into pTrc99A (17), grown in liquid medium, and mini-

mini-prepped to yield the library plasmid pTrc-[*luxR*] (Fig. 1A, square bracket indicates the *luxR* library). The library plasmid was then co-transformed with pAC-*Plux-gfp_{UV}* into *E. coli*. Approximately 60% of the clones retained 3OC6HSL-responsive fluorescence, whereas others (ca. 40%) showed a “constitutively-off” phenotype, implying a loss of function/folding capability by deleterious mutations.

E. coli was co-transformed with pTrc-[*luxR*] and pAC-*Plux-HSVtk*. To isolate *LuxR* mutants that responded to C8HSL, the pool of JW1226 harboring pTrc-[*luxR*] was subjected to ON-selection (see experimental section) in the presence of 100 nM C8HSL. The survivors were pooled, from which the mixture of plasmid DNA was isolated for further analysis.

C8HSL-sensitive *LuxR* variants

We isolated four *LuxR* variants with elevated sensitivity to C8HSL (Table 1). The sensitivity to C8HSL was acquired not by specificity shifting but rather by the overall elevated sensitivity, which was probably due to the elevated transition efficiency from the non-active to active forms. The quantitative liquid-phase characterization revealed that these variants had elevated sensitivity not only to C8HSL but also to all tested acyl-HSLs (the data for *LuxR_{C8-1}* and *LuxR_{C8-2}* are shown in Fig. 2B and C).

Sequence analysis revealed that all of the four variants shared the same amino acid substitution, C245W, which was the only non-synonymous mutation found in *LuxR_{C8-1}*

1) C245 is positioned at five amino acids from the carboxyl terminus of LuxR. It contains part of a DNA-bonding domain (residues 163-250), which is sufficient to activate gene expression in a ligand-independent manner (20). The residue C245 has never been addressed in relation to the function of LuxR. During the course of alanine-scan mutagenesis of the C-terminal residues (210-250), Trott and Stevens constructed a C245A LuxR mutant and found no significant difference in cellular function compared to wildtype (21). We created a saturation library of LuxR at this position and selected mutants with elevated sensitivity to C8HSL; all of the variants with bright fluorescence had a tryptophan at this position. In addition, we created and evaluated LuxR variants with the single mutations C245I, C245L, C245F, and C245Y by using site-directed mutagenesis. None of these mutants were more sensitive to HSLs than to WT, indicating that tryptophan is the only amino acid that confers this high-sensitivity phenotype on LuxR (Fig. S1). Thus, the presence of tryptophan, not the loss of cysteine at this position, is responsible for the elevated sensitivity.

In addition to C245W, LuxR_{C8-2} possesses four other mutations (Q27R, Y47H, N86K, and I110V) in the signal-binding domain. Of these mutations, we found that N86K alone increased LuxR sensitivity to various acyl-HSLs (Fig. 2D). Structure modeling indicated that N86 is located in the signal-binding domain but is not involved in binding to the acyl-HSLs (Fig. S2).

Although N86 and C245 are distant **in space** from each other, **the** single mutants at these residues (LuxR_{N86K} and LuxR_{C8-1}, respectively) were functionally **indistinguishable** (**Fig. 2B** and **D**). Interestingly, the combination of N86K and C245W resulted in a drastic increase in expression in the absence of signaling molecules (**Fig. 2E**). This *leakiness* was markedly suppressed by **any of the** other mutations (Q27R, Y47H or I110V), recovering the LuxR ON/OFF ratio (**Fig. 2C**).

The effect of C245W on the switching properties of LuxR mutants

Located far from the signal-binding domain, C245W **appeared to increase** the sensitivity of LuxRs without altering their signal preferences. We tested this hypothesis by introducing C245W into LuxR_{I58N} and LuxR_{Y62H}, **which are** specificity-switching mutants **that were** isolated in different directed evolution programs (they are 3OC6HSL-**s**pecific and 3OC8HSL-specific mutants, respectively) (**22**). As expected, the resultant LuxR_{I58N-C245W} and LuxR_{Y62H-C245W} showed higher sensitivity than **did** their respective parents **but maintained** their original signal preferences (**Fig. 3**).

Effective concentration of sensitive mutants

To understand how the C245**W** and M86**K** elevates the overall sensitivity of LuxR, we fused histidine hexamer tags to the N-termini of the LuxR variants and **performed** western blotting

of these variants (**Fig. 4**). Although LuxRs are known to fold better in the presence of acyl-acyl-HSLs (23), the effective cellular concentration of LuxR_{WT} appeared to be constant, regardless of the presence/absence of 3OC6HSL. In contrast, the effective cellular concentrations of mutants, especially for N86K-containing ones (LuxR_{C8-2} and LuxR_{N86K-LuxR_{N86K-C245W}}) were 2.8 times higher with the addition of 3OC6HSL.

Antagonistic/agonistic response to p-Coumaroyl homoserine lactone.

To test the possible antagonist activity of *p*-Coumaroyl homoserine lactone (*p*CHSL), we added varying concentrations of *p*CHSL and a fixed concentration (100 nM) of 3OC6HSL to *E. coli* harboring LuxR variants with pAC-*Plux-gfp_{UV}*. In the absence of *p*CHSL, all of the LuxRs were activated by its cognate signal, 3OC6HSL. As the concentration of *p*CHSL applied to the system increased, *E. coli* harboring LuxR_{WT} and LuxR_{C8-1} showed a decrease in GFP_{UV} fluorescence (**Fig. 5A**). Thus, *p*CHSL acts antagonistically (or in an inhibitory fashion) to LuxR and its variants. In contrast, LuxR_{C8-2} was completely free from the antagonistic effect of *p*CHSL in all tested concentrations (0-100 μM) (**Fig. 5A**).

To test the possible agonist function of *p*CHSL to LuxR variants, we added varying concentrations of *p*CHSL to *E. coli* expressing LuxR variants with pAC-*Plux-gfp_{UV}*. Neither LuxR_{WT} nor LuxR_{C8-1} responded to *p*CHSL at any of the tested concentrations (0–100 μM).

1 However, LuxR_{C8-2} was activated upon the addition of pCHSL (> 1 μM) (Fig. 5B). Thus,
2 pCHSL exerts agonist activity on LuxR_{C8-2}.

4 Discussion

5 *Molecular basis of elevation of the signal sensitivity*

6 LuxR requires a high concentration of C8HSL (> 1 μM) to be activated. Some LuxR
7 homologs, such as CepR from *Burkholderia cepacia*, are activated by C8HSL with high
8 sensitivity (EC₅₀ < 1 nM) (24). Collins *et al.* demonstrated that at least six different amino
9 acid substitutions could independently increase the C8HSL sensitivity of LuxR (8).

10 In the course of reproducing these data with selection-based directed evolution, we found
11 another mutation, C245W, that increased the sensitivity of LuxR variants without changing
12 their signaling preferences (Figs. 2 and 3). Interestingly, none of the previously described
13 C8HSL-sensitizing mutations (such as T33A, S116A, and M135I) (8) were found among the
14 LuxR variants that we isolated. Considering the library size (~10⁶) of LuxR that we
15 it is unlikely that none of these mutations existed in the original pool before selection. If the
16 pool actually harbored variants with those mutations, it follows that they were outcompeted
17 by C245 mutants during the selection process. We do not know the exact mechanism by
18 which this occurred. Notably, we conducted ON-selection in media containing 100 nM of
19 C8HSL, whereas the previous C8HSL-sensitive mutations were found by GFP screening on

1 a plate containing 200 nM of C8HSL (8). Indeed, the previous work failed to find any
2 fluorescent clones on the screening plate containing 50 nM C8HSL (8). Why was C245W
3 found in the previous work? Here again, we do not know exactly. It could possibly be related
4 to the fact that the nucleotide sequence encoding this residue was within the annealing site
5 of PCR primers used for library construction (8). The annealing sites of PCR primers are
6 significantly lower in mutation rates during error-prone PCR (16).

7 Residue 245 is five amino acids upstream from the carboxyl terminus of LuxR. The roles
8 of the LuxR C-terminal domain are DNA binding and recruiting RNA polymerase, and it is
9 not directly involved in signal recognition (21, 25, 26). Thus, it is unlikely that C245W
10 directly increases the binding affinity of LuxR to C8HSL.

11 We found that the N86K mutation also confers a C245W-like phenotype to LuxR (Fig. 2C
12 and D). Because the N-terminal domain inhibits the DNA binding of the C-terminal domain
13 the absence of acyl-HSL (27), it is tempting to hypothesize that both N86 and C245 are
14 deeply involved in this switching machinery. Mutations in these residues may partially
15 the interaction between the domains, thereby facilitating the transition from the inactive form
16 into the active form. It should be noted that all isolated mutants with higher signaling
17 sensitivity showed elevated expression levels, especially in the presence of HSLs (Fig. 4).
18 Because LuxR is more stable and soluble in its active, HSL-bound form (23), C245W
19 probably confers the overall stability of LuxR via elevated efficacy in the transition into the

active soluble form. This “quick-and-dirty” acquisition of signal sensitivity blurred the gap between the inactive and active forms, resulting in a decrease in stringency (**Fig. 2C** and LuxR mutants with both the C245W and N86K mutations exhibited increased leaky expression in signal-free conditions and an elevated effective concentration in the absence of HSLs (**Fig. 5**).

Single mutation converts LuxR antagonist into agonist

*p*CHSL is the cognate signal in the quorum sensing system of *Rhodopseudomonas palustris* (28). However, *p*-Coumaric acid is an antagonist to various other LuxR homologs (29). Many HSLs with aromatic side chains are also antagonists of LuxR (30, 31). We found that *p*CHSL was indeed an antagonist to LuxR_{WT} and its variants (**Fig. 5A**). However, we also found that it activates LuxR_{C8-2} (**Fig. 5B**). By creating and testing the single mutants for each of the five mutations found in LuxR_{C8-2}, we found that N86K was responsible for the agonistic response to *p*CHSL (**Fig. S3**).

It should be noted that LuxR_{C8-2} was obtained in the selection scheme for elevated sensitivity to weak agonist C8HSL, not in the direct selection for an agonistic response to *p*CHSL. Indeed, LuxR_{C8-1}, another variant obtained in the same selection, was antagonized by *p*CHSL. Thus, the border between the activator (agonist) and inhibitor (antagonists) is quite blurry, especially in an evolutionary context. If this holds true for other LuxR homologs,

any antagonists of LuxR homologs (30, 32) could easily be neutralized or converted into agonists (13) during the non-directional divergence of the receptors, even without counter-counter-selection. This also partially explains why quorum sensing systems can undergo rapid diversification in their signaling sensitivity and exhibit the frequent emergence of orthogonality and cross-inhibition (33, 34).

Expanding the cellular communication toolbox

Synthetic biologists have extensively utilized LuxR family proteins to implement artificial cell-to-cell communications and coordinate the behavior of cell populations (3, 6, 7, 35). To reliably assemble the signaling networks, especially those with complexity, an on-demand but fast-tracked construction of LuxR variants with exact required specifications would be helpful. We found that the mutation C245W increased the general sensitivity of LuxR without affecting its signal preference (**Fig. 3**). Such “modularity” of mutational effects is desirable because one can quickly and predictably combine them into LuxR with specificities required for various unique contexts.

In the laboratory evolution of C8HSL-sensitivity, LuxR acquired mutations that altered its efficiency in the transition to the active form upon the binding of each acyl-HSL, rather than those that directly altered the binding affinity between LuxR and acyl-HSLs. This was achieved by shifting the equilibrium between the inactive/active forms by increasing the

1 stability of the LuxR ligand-bound (active) state. It has been long recognized that LuxR (36,
2 37) and its homologs (20, 38, 39, 40) are stabilized upon binding with HSLs and are
3 protected from proteolytic degradation in the cell. As a result, the effective cellular
4 concentration of active-form LuxR is elevated (**Fig. 4**). This type of mutation may be one of
5 the most accessible and, therefore, prevailing molecular solutions during the course of
6 divergent evolution LuxR proteins. It would be of interest to determine how and to what
7 extent this type of evolutionary intermediate can diverge and whether it can quickly evolve
8 orthogonality and cross-inhibition, as is frequently seen in nature (33) (34).

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References

1. **Churchill, M. E. and Chen, L.:** Structural basis of acyl-homoserine lactone-dependent signaling, *Chem Rev*, **111**, 68-85 (2011).
2. **Steindler, L. and Venturi, V.:** Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors, *FEMS Microbiol Lett*, **266**, 1-9 (2007).
3. **Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., and Weiss, R.:** A synthetic multicellular system for programmed pattern formation, *Nature*, **434**, 1130-1134 (2005).
4. **Collins, C. H., Leadbetter, J. R., and Arnold, F. H.:** Dual selection enhances the signaling specificity of a variant of the quorum-sensing transcriptional activator LuxR, *Nat Biotechnol*, **24**, 708-712 (2006).
5. **You, L., Cox, R. S., 3rd, Weiss, R., and Arnold, F. H.:** Programmed population control by cell-cell communication and regulated killing, *Nature*, **428**, 868-871 (2004).
6. **Brenner, K., Karig, D. K., Weiss, R., and Arnold, F. H.:** Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium, *Proc Natl Acad Sci U S A*, **104**, 17300-17304 (2007).
7. **Tamsir, A., Tabor, J. J., and Voigt, C. A.:** Robust multicellular computing using genetically encoded NOR gates and chemical 'wires', *Nature*, **469**, 212-215 (2011).
8. **Collins, C. H., Arnold, F. H., and Leadbetter, J. R.:** Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones, *Mol Microbiol*, **55**, 712-723 (2005).
9. **Hawkins, A. C., Arnold, F. H., Stuermer, R., Hauer, B., and Leadbetter, J. R.:** Directed evolution of *Vibrio fischeri* LuxR for improved response to butanoyl-homoserine lactone, *Appl Environ Microbiol*, **73**, 5775-5781 (2007).
10. **Fuqua, C., Parsek, M. R., and Greenberg, E. P.:** Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing, *Annu Rev Genet*, **35**, 439-468 (2001).
11. **Zhang, R. G., Pappas, K. M., Brace, J. L., Miller, P. C., Oulmassov, T., Molyneaux, J. M., Anderson, J. C., Bashkin, J. K., Winans, S. C., and Joachimiak, A.:** Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA, *Nature*, **417**, 971-974 (2002).
12. **Volpari, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P., and Marco, S. D.:** The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA, *EMBO J*, **21**, 4393-4401 (2002).
13. **Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S., and Givskov, M.:** The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors, *Microbiology*, **151**, 3589-3602 (2005).
14. **Tashiro, Y., Fukutomi, H., Terakubo, K., Saito, K., and Umeno, D.:** A nucleoside kinase as a dual selector for genetic switches and circuits, *Nucleic Acids Res*, **39**, e12 (2011).
15. **Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H.:** Construction of *Escherichia coli* K-12 in-frame, single-gene knockout

- mutants: the Keio collection, *Mol Syst Biol*, **2**, 2006 0008 (2006).
16. **Cirino, P. C., Mayer, K. M., and Umeno, D.:** Generating mutant libraries using error-prone PCR, *Methods Mol Biol*, **231**, 3-9 (2003).
 17. **Amann, E., Brosius, J., and Ptashne, M.:** Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*, *Gene*, **25**, 167-178 (1983).
 18. **Schaefer, A. L., Hanzelka, B. L., Eberhard, A., and Greenberg, E. P.:** Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs, *J Bacteriol*, **178**, 2897-2901 (1996).
 19. **Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P., and Stewart, G. S.:** Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing, *FEMS Microbiol Lett*, **163**, 185-192 (1998).
 20. **Choi, S. H. and Greenberg, E. P.:** The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain, *Proc Natl Acad Sci U S A*, **88**, 11115-11119 (1991).
 21. **Troen, A. E. and Stevens, A. M.:** Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*, *J Bacteriol*, **183**, 387-392 (2001).
 22. **Tashiro, Y., Kimura, Y., Furubayashi, M., Tanaka, A., Terakubo, K., Saito, K., Kawai-Noma, S., and Umeno, D.:** Directed evolution of autoinducer selectivity of *Vibrio fischeri* LuxR, *J Gen Appl Microbiol*, in press (2016).
 23. **Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S., and Givskov, M.:** Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover, *Microbiology*, **148**, 1119-1127 (2002).
 24. **Weingart, C. L., White, C. E., Liu, S., Chai, Y., Cho, H., Tsai, C. S., Wei, Y., Delay, N. R., Gronquist, M. R., Eberhard, A., and Winans, S. C.:** Direct binding of the quorum sensing regulator CepR of *Burkholderia cenocepacia* to two target promoters *in vitro*, *Mol Microbiol*, **57**, 452-467 (2005).
 25. **Egland, K. A. and Greenberg, E. P.:** Quorum sensing in *Vibrio fischeri*: analysis of the LuxR DNA binding region by alanine-scanning mutagenesis, *J Bacteriol*, **183**, 382-386 (2001).
 26. **Finney, A. H., Blick, R. J., Murakami, K., Ishihama, A., and Stevens, A. M.:** Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the *lux* operon during quorum sensing, *J Bacteriol*, **184**, 4520-4528 (2002).
 27. **Urbanowski, M. L., Lostroh, C. P., and Greenberg, E. P.:** Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein, *J Bacteriol*, **186**, 631-637 (2004).
 28. **Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Juhaszova, K., Sufrin, J. R., and Harwood, C. S.:** A new class of homoserine lactone quorum-sensing signals, *Nature*, **454**, 595-599 (2008).
 29. **Bodini, S. F., Manfredini, S., Epp, M., Valentini, S., and Santori, F.:** Quorum sensing inhibition activity of garlic extract and *p*-coumaric acid, *Lett Appl Microbiol*, **49**, 551-555 (2009).
 30. **Reverchon, S., Chantegrel, B., Deshayes, C., Doutheau, A., and Cotte-Pattat, N.:** New synthetic

analogues of N-acyl homoserine lactones as agonists or antagonists of transcriptional regulators involved in bacterial quorum sensing, *Bioorg Med Chem Lett*, **12**, 1153-1157 (2002).

31. **Geske, G. D., O'Neill, J. C., Miller, D. M., Wezeman, R. J., Mattmann, M. E., Lin, Q., and Blackwell, H. E.:** Comparative analyses of *N*-acylated homoserine lactones reveal unique structural features that dictate their ability to activate or inhibit quorum sensing, *Chembiochem*, **9**, 389-400 (2004).

32. **Song, K. M., Bu, Y., and Suga, H.:** Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs, *Chem Biol*, **10**, 81-89 (2003).

33. **Elder, A.:** Social conflict drives the evolutionary divergence of quorum sensing, *Proc Natl Acad Sci U S A*, **108**, 13635-13640 (2011).

34. **Pollak, S., Omer-Bendori, S., Even-Tov, E., Lipsman, V., Bareia, T., Ben-Zion, I., and Eldar, A.:** Facultative cheating supports the coexistence of diverse quorum-sensing alleles, *Proc Natl Acad Sci U S A*, **113**, 2152-2157 (2016).

35. **Danino, T., Mondragon-Palomino, O., Tsimring, L., and Hasty, J.:** A synchronized quorum of genetic clocks, *Nature*, **463**, 326-330 (2010).

36. **Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S., and Givskov, M.:** Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover, *Microbiology*, **148**, 1119-1127 (2002).

37. **Urbanowski, M. L., Lostroh, C. P., and Greenberg, E. P.:** Reversible Acyl-Homoserine Lactone Binding to Purified *Vibrio fischeri* LuxR Protein, *Journal of Bacteriology*, **186**, 631-637 (2004).

38. **Zhu, J. and Winans, S. C.:** The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization, *Proc Natl Acad Sci U S A*, **98**, 1507-1512 (2001).

39. **Nguyen, Y., Nguyen, N. X., Rogers, J. L., Liao, J., MacMillan, J. B., Jiang, Y., and Sperandio, V.:** Structural and mechanistic roles of novel chemical ligands on the SdiA quorum-sensing transcription regulator, *MBio*, **6**, e02429-02414 (2015).

40. **Kim, T., Duong, T., Wu, C., Choi, J., Lan, N., Kang, S. W., Lokanath, N. K., Shin, D., Hwang, H.-Y., and Kim, K. K.:** Structural insights into the molecular mechanism of *Escherichia coli* SdiA, a quorum-sensing receptor, *Acta Crystallogr D Struct Biol*, **D70**, 694-707 (2014).

Figure/Table Captions

Fig. 1. Directed evolution of LuxR for altered sensitivity. A. Plasmid construction and acyl-HSLs used in this work. **B.** Genetic selection of LuxR mutants that can respond to C8HSL with improved sensitivity.

Fig. 2. Activation of *Plux-gfp_{UV}* with 3OC6HSL (filled diamonds), 3OC8HSL (filled circles), C6HSL (open diamonds), and C8HSL (open circles) by LuxR variants. The data were normalized to the optical density and corrected by subtracting the background fluorescence. All measurements were performed in triplicate. The error bars indicate the standard deviation.

Fig. 3. The effect of C245W on the sensitivity of LuxR derivatives to different signals. Two specificity variants of LuxR ((**A**) **LuxR_{I58N}** and (**B**) **LuxR_{H62K}**) with (filled circle) and without (open circle) C245W were constructed and tested their response to 3OC6HSL, 3OC8HSL, C6HSL, and C8HSL. The data were normalized by the optical density and corrected by subtracting the background fluorescence. The error bars indicate the standard deviation of three independent experiments.

Fig. 4. Western blot analysis of LuxR variants. From cells harboring His-tagged LuxR variants, the total and soluble fractions from approximately 10^8 cells were obtained in the presence (1 μ M) and absence of 3OC6HSL. The spots were stained with HRP-conjugated anti-His-tag antibody.

Fig. 5. Dose response of LuxR variants (filled diamonds: LuxR_{WT}, filled circles: LuxR_{C8-1}, filled triangles: LuxR_{C8-2}) to pCHSL. The effect of pCHSL on LuxR variants was studied by measuring the expression level of the reporter (*gfp_{UV}*) at varying concentrations of pCHSL in the presence of near-saturation concentration (**A**: 100 nM) or absence (**B**) of 3OC6HSL.

Table 1. The LuxR variants found/ used in this work.

1

2 **Table 1. The LuxR variants found/ used in this work.**

Entry number and name		Mutations
1	LuxR _{C8-1}	C735G (C245W)
2	LuxR _{C8-2}	A80G (Q27R) T139C (Y47H) C258A (N86K) A328G (I110V) C735G (C245W)
3	LuxR _{C8-3}	A545G (D182G) C735G (C245W) A748G (N250D)
4	LuxR _{C8-4}	A50G (K17M) G734T (C245F)

3

Fig 1

Fig 2

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