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Full Paper

Directed evolution of the autoinducer selectivity of *Vibrio fischeri* LuxR

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LuxR family transcriptional regulators are the core components of quorum sensing in Gram-negative bacteria and exert their effects through binding to the signaling molecules acyl-homoserine lactones (acyl-HSLs). The function of the LuxR homologs is remarkably plastic, and naturally occurring acyl-HSLs are structurally diverse. To investigate the molecular basis of the functional plasticity of *Vibrio fischeri* LuxR, we directed the evolution of LuxR toward three different specificities in the laboratory. We found an orthogonal pair of LuxR mutants specific either to 3-oxo-hexanoyl homoserine lactone or to 3-oxo-octanoyl homoserine lactone. Interestingly, the majority of the specificity changes did not arise from modulating the recognition event but rather from changing the efficiency of the transition from the inactive form to the active form upon signal binding. This finding explains how quorum sensing systems can rapidly diverge in nature and in the laboratory and how signal orthogonality and mutual inhibition frequently occur among closely related diverging systems.

Key Words: agonist; antagonist; orthogonal system; quorum sensing; synthetic biology; transcription factor

Introduction

Quorum sensing is a microbial cell-to-cell communication system that monitors cell population density and regulates a diverse array of physiological activities (e.g., Atkinson and Williams, 2009; Bassler and Losick, 2006; Fuqua and Greenberg, 2002). Due to their critical role in regulating virulence genes, molecules involved in quorum sensing (signal molecules, producers, and receptors) have been studied as important medical targets (e.g., Njoroge and Sperandio, 2009). In addition, these natural signaling systems have been used by biological engineers to construct bacterial sensors (e.g., Steindler and Venturi, 2007), artificial bacterial consortiums (e.g., Basu et al., 2005; Brenner et al., 2007; Collins et al., 2006; Regot et al., 2011; Tamsir et al., 2011; You et al., 2004), and population synchronizers (e.g., Chen et al., 2015; Danino et al., 2010).

In Gram-negative bacteria, acyl-homoserine lactones (acyl-HSLs) are the signaling molecules for quorum sensing (e.g., Churchill and Chen, 2011). Acyl-HSLs are synthesized by the LuxI protein family, transported across the bacterial cell wall, and recognized by sensor proteins (the LuxR protein family) both in other bacteria and in their producers. Upon binding to acyl-HSLs, the sensory proteins undergo a structural transition, binding to or releasing a specific DNA sequence (*lux* box) (e.g., Fuqua et al., 2001) and mediating gene expression under the control of this sequence.

To date, dozens of natural HSL signals have been identified, and there are LuxR homologues that respond to each of those HSLs as cognate signals. LuxR family proteins can recognize various non-cognate acyl-HSLs, implying a significant level of cross-species communication (e.g., Ahmer, 2004; Bassler and Losick, 2006; Fuqua et al., 2001). Each bacterium possesses a unique "spectrum" of sensitivity to acyl-HSLs, which are suited to its living environment. Some bacterial species even show intra-species diversity in LuxI/LuxR systems, and a signal from one strain can activate its own receptor but fail to activate (or even inhibit) a closely related receptor from a different strain (e.g., Chatterjee et al., 2005; Morohoshi et al., 2008).

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A notable example is *Erwinia carotovora*, for which one subspecies specifically responds to 3-oxo-octanoyl homoserine lactone (3OC8HSL) but not to 3-oxo-hexanoyl homoserine lactone (3OC6HSL), whereas another subspecies specifically responds to 3OC6HSL but not to 3OC8HSL (e.g., Chatterjee et al., 2005). This requires the establishment of a signaling orthogonality between their sensory proteins ExpRs. Diversifying selection has likely driven the formation of the various types of acyl-HSLs present in nature (e.g., Ansaldi et al., 2002; Ichihara et al., 2006). Even so, it is still surprising that such complete orthogonality between ExpR variants was achieved in a short period of time, considering the subtle structural difference between these two signals.

In this work, we describe an effort to reproduce this in the laboratory. Using the powerful selection system tailored for genetic switches (e.g., Tashiro et al., 2011), we aimed to isolate 3OC6-specific and 3OC8-specific mutants from a single library of LuxR. Analysis of the isolated variants revealed some key residues that govern the specificity of signaling. Additionally, we show that the changes in function arose primarily, if not exclusively, from changes in the efficiency of transition to an active form, rather than changes in the molecular recognition profile via reshaping of the signal-binding pocket.

Materials and Methods

Bacterial strains, media, and growth conditions. *E. coli* strains DH10B (F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*)Φ80d*lacZ*ΔM15 Δ*lac*X74*recA1 endA1 araD*139 Δ(*ara*, *leu*)7697 *galU galK* λ^- *rpsL* (Str^R) *nupG*) (Invitrogen, CA) and XL10 Gold (Tet^R Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi*-1 *recA1 gyrA96 relA1 lac* Hte [F′*proAB lacI*^{q}ZΔM15 Tn*10* (Tet^R) Tn5 Kan^R Amy]) (Stratagene, CA) were used for cloning and library construction. JW1226 (F^- Δ(*araD-araB*)567 Δ*lacZ*4787(::*rrnB*-3) λ^- Δ*tdk*-747::*kan rph*-1 Δ(*rhaD-rhaB*)568, *hsdR*514) from the KEIO collection (Baba et al., 2006), which harbored pAC(P*lux*)-gfp_{UV} or pAC(P*lux*)-HSV*tk*, was used for GFP screening and for genetic selection, respectively. *E. coli* strains were incubated in LB medium (2.0%(w/v) 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 μg ml^{-1} chloramphenicol (Nacalai Tesque) and/or 50 μg ml^{-1} carbenicillin (Invitrogen).

The acyl-HSLs used in this study were 3OC6HSL, 3OC8HSL, C6HSL, and C8HSL. 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 stored at –20°C. The stock solutions of pC-HSL were prepared with ethanol instead of ethyl acetate (1–10 mM). For liquid-phase experiments, we dispensed acyl-HSL stock solutions into autoclaved 96-well 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 reading frame of *luxR* was PCR-cloned into the *Nco*I/*Hin*dIII 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*) (Tashiro et al., 2011) were PCR-cloned into the *Nco*I/*Hin*dIII site of pTrc99A. The resultant pTrc-gfp_{UV} and pTrc-HSV*tk* were then PCR-cloned and subcloned into *Cla*I/*Hin*dIII, 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′-ACCTGTAGGATCGTACAGGTTTACGCAAGAAAA-TGGTTTGTTATAGTCGAATAAA-3′), resulting in pAC(P*lux*)-HSV*tk* 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 μM concentration of each primer (primer 1: 5′-CAATCTGTGTGGGCACTCGAC-3′ and primer 2: 5′-TACTGCCGCCAGGCAAATTC-3′). The reaction mixtures (50 μl total volume) contained 5 U of Taq DNA polymerase (New England Biolabs, MA), 200 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, and 10 μM $MnCl_2$, as previously described by Cirino et al. (2003). The amplification factor was approximately 1,000 (approximately 10 effective rounds). The PCR product was subcloned into the *Nco*I/*Hin*dIII 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 ≈ 10^6).

Selection and screening process. The *luxR* library was introduced into JW1226 harboring pAC(P*lux*)-HSV*tk*, and the cells were cultured overnight in 10 ml LB medium containing the appropriate type and concentration of acyl-HSLs. For each of the three selection programs, the transformants (≈10^6 cells) from the culture were inoculated into 1 ml of OFF-selection medium (LB medium including 1 μM dP; Berry & Associates, MI) including the same concentration and type of acyl-HSL as the preculture, followed by shaking for 2 h at 37°C (OFF-selection). The cell culture was washed twice with the same volume of LB medium containing a different acyl-HSL. After 6 h, a portion of the cells (containing ≈ 10^6 cells) was inoculated into ON-selection medium (2.0% (w/v) tryptone; Becton, Dickinson and Company, MD, 0.5%(w/v) NaCl; Nacalai Tesque, 10 μg ml^{-1} thymidine; Wako, JP, 1 μg ml^{-1} adenosine; Tokyo Chemical Industry Co., LTD., JP, and 20 μg ml^{-1} 2′-deoxy-5-fluorouridine; Sigma-Aldrich) and incubated for 20 h (ON-selection). After one round of OFF- and one round of ON-selections, plasmid DNA was isolated from the selected culture.

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 using self-ligation. The ligation products were transformed into XL10 Gold, plated

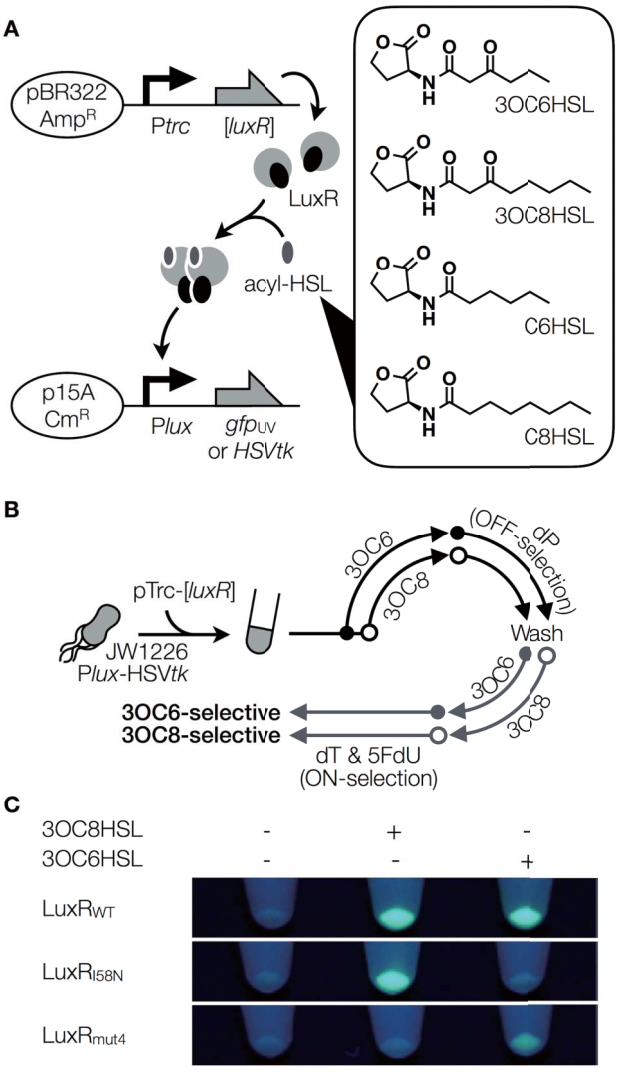

Fig. 1. Directed evolution of LuxR for altered specificity and sensitivity.

A. Plasmid construction and HSLs used in this work. B. Genetic selection of LuxR mutants. Shown is the schematic illustration of the process of isolating the LuxR mutants that can distinguish 3OC8HSL from 3OC6HSL. C. Selective activation of LuxR variants using either 3OC6HSL or 3OC8HSL. *E. coli* JW1226 harboring pAC(P*lux*)-*gfp*$_{UV}$ was transformed with pTrc-*luxR*wt, pTrc-*luxR*$_{I58N}$, or pTrc-*luxR*$_{mut4}$. After 12 h growth in LB medium with 500 nM 3OC6HSL, 500 nM 3OC8HSL, or no acyl-HSL, the cells were centrifuged to form a pellet that was photographed under illumination by near-UV light.

on LB-agar, and grown overnight to isolate clones.

Similarly, site-saturation mutagenesis was induced for I58 and Y62 using ExSite PCR with the primers containing the 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.

Gene expression analysis using GFP$_{UV}$ as a reporter. For rapid screening, the library plasmids were transformed into JW1226 containing pAC(P*lux*)-*gfp*$_{UV}$ and incubated on LB agar plates overnight. These colonies were picked and inoculated into 500 μl LB medium in 96-well deep-well

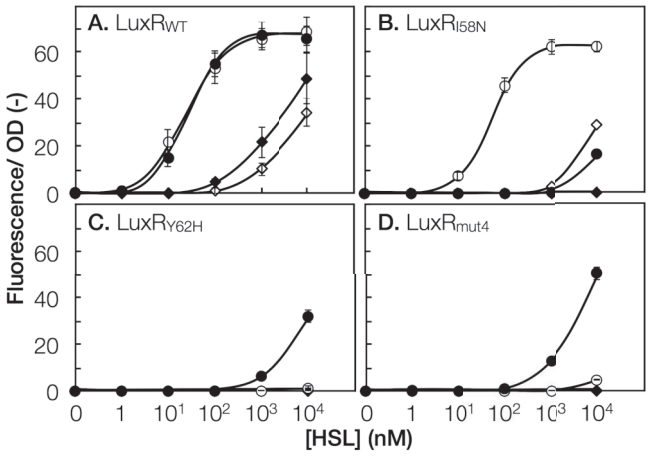

Fig. 2. Activation of P*lux*-*gfp*$_{UV}$ with 3OC6HSL (closed circles), 3OC8HSL (open circles), C6HSL (closed diamonds), and C8HSL (open diamonds) by LuxR variants.

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.

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(P*lux*)-*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 μl 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 μl each) were diluted 10-fold into fresh LB medium (a total volume of 200 μl) 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.

Results

LuxR-Plux system for acyl-HSL detection

First, we verified the test system for LuxR sensitivity to various acyl-HSLs. For this purpose, we introduced two plasmids into an *E. coli* strain JW1226 (Fig. 1A). One plasmid, pTrc-*luxR*, encodes the *luxR* gene under the *trc* promoter (e.g., Amann et al., 1983). The second plasmid, pAC(P*lux*)-*gfp*$_{UV}$, contains the *gfp*$_{UV}$ gene under the control of the *lux* promoter. Co-transformation with these two plasmids allows cells to fluoresce in response to acyl-HSLs. In this system, wild-type LuxR required approximately 25 nM 3OC6HSL to achieve a half-maximal level of gene activation (EC$_{50}$) (Fig. 2A). LuxR$_{WT}$ was also activated by 3OC8HSL with a similar sensitivity (EC$_{50}$ ≈ 20 nM). LuxR was activated by acyl-HSLs lacking a 3-oxo group (hexanoyl homoserine lactone (C6HSL) and

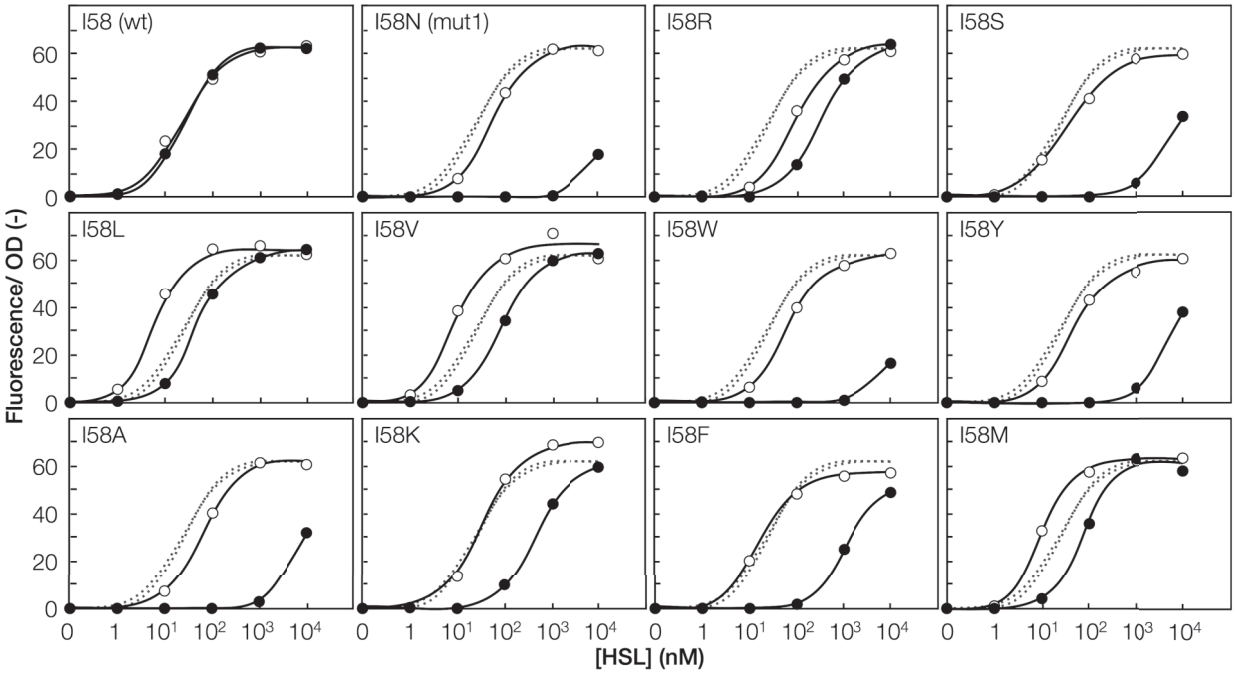

Fig. 3. The effect of amino acid substitution at I58 on the specificity of LuxR.

Plasmids encoding LuxRs were introduced into *E. coli* JW1226 harboring pAC(P*lux*)-gfp_{UV} to measure the transfer function of 3OC6HSL (closed circles) and 3OC8HSL (open circles). Dashed lines represent the wild-type response to these signaling molecules. The data were normalized to the optical density and corrected by subtracting the background fluorescence.

octanoyl homoserine lactone(C8HSL)), but only in the presence of high concentrations of these compounds (EC_{50} > 1 μM). These results were in good agreement with previous reports (e.g., Schaefer et al., 1996; Winson et al., 1998).

Construction of a whole-gene luxR library

We conducted PCR mutagenesis (e.g., Cirino et al., 2003) on the whole reading frame (750 nt, 250 aa) of LuxR. The resultant *luxR* library was ligated into pTrc99A (e.g., Amann et al., 1983), grown in liquid medium, and miniprepped to yield the library plasmid pTrc-[*luxR*] (Fig. 1A, the square bracket stands for the *luxR* library). The library plasmid was then co-transformed with pAC(P*lux*)-gfp_{UV} into JW1226. Approximately 60% of the clones retained 3OC6HSL-responsive fluorescence. The rest of the clones (approximately 40%) showed a *constitutively-off* phenotype, implying a loss of function/folding capability by deleterious mutations. There are several mutations that confer a *constitutively-on* phenotype (activating P*lux* even in the absence of acyl-HSLs) (e.g., Sitnikov et al., 1996), but we did not observe such clones in our library.

Parallel evolution of LuxR

We previously reported a selection system for genetic circuits (e.g., Tashiro et al., 2011). To explore the variability of LuxR specificity, we conducted two independent directed evolution programs (Fig. 1B).

E. coli was co-transformed with pTrc-[*luxR*] and pAC(P*lux*)-HSV*tk*. To isolate LuxR mutants that respond to 3OC8HSL but not to 3OC6HSL, the pool of JW1226 harboring pTrc-[*luxR*] was subjected to OFF-selection in the presence of 100 nM 3OC6HSL. After washing the cell with fresh LB medium, the resultant culture was diluted into ON-selection medium containing 100 nM 3OC8HSL (see Section "Materials and Methods" for details). To isolate LuxR variants with reversed specificity, the same library was first subjected to OFF-selection in the presence of 100 nM 3OC8HSL and then subjected to ON-selection in the presence of 100 nM 3OC6HSL. From each of two sets of the selection programs, the survivors were pooled, and their DNA was isolated for further analysis.

3OC8HSL-specific LuxR mutants

We isolated two variants from the pool subjected to OFF-selection in the presence of 3OC6HSL and to ON-selection in the presence of 3OC8HSL ($LuxR_{mut1}$ and $LuxR_{mut2}$ in Table S1). These variants showed a drastic decrease in sensitivity to 3OC6HSL but showed wild-type sensitivity to 3OC8HSL (data not shown). Because they shared the same amino acid substitution, I58N (Table S1), we created the LuxR variant with a single I58N mutation ($LuxR_{I58N}$, Fig. 1C). $LuxR_{I58N}$ exhibited wild-type sensitivity to 3OC8HSL but was approximately 1,000 times less sensitive to 3OC6HSL than LuxR (Fig. 2B).

3OC6HSL-specific LuxR mutants

To isolate LuxR variants with a preference for 3OC6HSL over 3OC8SHL, the library of LuxR was subjected to OFF-selection in the presence of 100 nM of 3OC8HSL followed by ON-selection in the presence of 100 nM 3OC6HSL. This time, very few clones survived the selection. From the survivors, we identified a mutant that minimally responds to 3OC8HSL ($LuxR_{mut3}$). It contained two nonsynonymous mutations, Y62H and N88Y (Table S1). By creating and testing both single mutants, we concluded that Y62H is responsible for the 3OC6HSL-specific phenotype (data not shown). The single mutant $LuxR_{Y62H}$ was

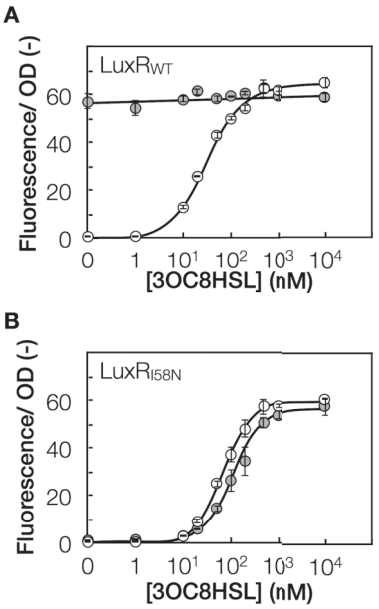

Fig. 4. Effect of 3OC6HSL on the response of $LuxR_{WT}$ and $LuxR_{I58N}$ to 3OC8HSL.

A plasmid encoding wild-type $LuxR_{WT}$ (A) or $LuxR_{I58N}$ (B) was introduced into *E. coli* JW1226 harboring pAC(P*lux*)-gfp_{UV} for the measurement of the 3OC8HSL-dependent activation of the *lux* promoter in the presence (gray circles) and absence (open circles) of 3OC6HSL (100 nM for (A) and 500 nM for (B)). The data were normalized to the optical density and corrected by subtracting the background fluorescence. All measurements were performed in triplicate, and the error bars indicate the standard deviation.

completely insensitive to 3OC8HSL (Fig. 2C). However, $LuxR_{Y62H}$ was also less sensitive than LuxRwt to 3OC6HSL and other acyl-HSLs. Site-saturation mutagenesis at position 62 revealed that two other amino acids, Gly and Asp, confer a similar 3OC6HSL specificity to $LuxR_{Y62H}$. On agar plates, cells harboring $LuxR_{Y62G}$ showed higher maximum levels of fluorescent protein than those expressing $LuxR_{Y62H}$ (data not shown).

$LuxR_{Y62H}$ was subjected to another round of random mutagenesis followed by visual screening for a higher level of fluorescence by P*lux*-gfp_{UV} in the presence of 3OC6HSL. We isolated four variants ($LuxR_{mut4–7}$ Table S1) that retained 3OC6HSL specificity and showed slightly higher maximum levels of gfp_{UV} expression (Fig. 2D).

The cellular functions of two LuxR mutants with altered signal specificities ($LuxR_{I58N}$ and $LuxR_{mut4}$) were compared. Plasmids expressing these mutants were cotransfected into *E. coli* with the plasmid coding gfp_{UV} under the control of the *lux* promoter. While the cells harboring $LuxR_{WT}$ fluoresced with the addition of 3OC6HSL or 3OC8HSL (Fig. 1C), the cells harboring $LuxR_{I58N}$ fluoresced only in the presence of 3OC8HSL, but not in the presence of 3OC6HSL. The opposite behavior was observed for the cells harboring $LuxR_{mut4}$; they appeared fluorescent only in the presence of 3OC6HSL.

Site saturation at I58

To further investigate how this residue determines signal specificity, we substituted I58 with various amino acids (Fig. 3). The apparent sensitivity of LuxR to 3OC8HSL remained approximately the same, irrespective of the type of amino acid situated at this position. In sharp contrast, the sensitivity of LuxR to 3OC6HSL decreased by substituting I58 with any other amino acid. Especially with the amino acids A, N, S, Y, F, or W at this position, LuxR showed virtually no response to 3OC6HSL.

3OC6HSL reduces sensitivity of $LuxR_{I58N}$ to 3OC8HSL

We compared the transfer function of $LuxR_{WT}$ and $LuxR_{I58N}$ in the presence and absence of a fixed concentration of 3OC6HSL (Fig. 4). In the presence of the cognate signal molecule 3OC6HSL, $LuxR_{WT}$ is fully activated, irrespective of the concentration of 3OC8HSL (Fig. 4A). By contrast, $LuxR_{I58N}$ was almost completely insensitive to 3OC6HSL, allowing the observation of a 3OC8HSL-dependent transfer curve in both its presence and absence (Fig. 4B). We found that the apparent sensitivity of $LuxR_{I58N}$ to 3OC8HSL was reduced by the addition of 3OC6HSL: the EC_{50} value in the presence of 3OC6HSL (140 nM) was twice as high as that in its absence (70 nM). This parallel shift of the dose-response curve is the typical indicator that 3OC6HSL acts as an antagonist (e.g., Vauquelin et al., 2002) to LuxR. We also compared $LuxR_{WT}$ and $LuxR_{I58N}$ in their dose-response to 3OC6HSL in the presence of a fixed concentration (100 nM) of 3OC8HSL (Fig. S1). The level of $LuxR_{WT}$ activation increased with the concentration of 3OC6HSL, whereas that of $LuxR_{I58N}$ declined with the concentration of 3OC6HSL.

Thus, 3OC6HSL weakly inhibits the binding of 3OC8HSL of $LuxR_{I58N}$. This finding clearly indicates that $LuxR_{I58N}$ retains significant affinity for 3OC6HSL, despite its loss of sensitivity to the molecule (Fig. 2B).

Discussion

I58N selectively abolishes the response to acyl-HSLs with C6 side chains

Amino acid substitutions in I58 result in a drastic decrease in sensitivity to 3OC6HSL and C6HSL, but preserve sensitivity to acyl-HSLs with larger (e.g., 3OC8HSL and C8HSL) side chains (Fig. 3). It remains unclear exactly how this mutation results in a selective loss of sensitivity to the smaller signals. In the LuxR homolog TraR, residue A49 corresponds to residue 58 in LuxR (e.g., Whitehead et al., 2001). In the TraR structure, A49 is in close proximity to the acyl-HSL side chains. The replacement of A49 with I or M resulted in a selective decrease in the sensitivity to 3OC8HSL, but the response to an acyl-HSL with shorter (3OC6) side chains was preserved (e.g., Chai and Winans, 2004). They argued that substitution of A49 with bulkier amino acids (I and M) may establish a steric barrier to C8- or longer side chains (e.g., Chai and Winans, 2004). Interestingly, I58N in LuxR has exactly the opposite effect. We found that replacement of I58 with A, N, S, Y, F, or W resulted in a nearly complete loss of response to 3OC6HSL (Fig. 3). No apparent physical property (e.g., size, pI, and polarity) is shared by these six amino acids, and we cannot propose a simple structural model explaining how this residue selectively modulates sensitivity to 3OC6HSL.

Although designing steric blockers in the correct pocket

position can effectively inhibit the binding of larger signals (3OC8HSL) (Chai and Winans, 2004), it is not easy to formulate a blockage binding selective inhibitor for the smaller signals (3OC6HSL). From the viewpoint of molecular recognition, reshaping the binding pocket to selectively exclude smaller molecules appears to require multiple procedures. Nevertheless, a single mutation sometimes results in the selective loss of the sensitivity to smaller molecules. For instance, Luo et al. (2003) reported a similar finding in TraR: A105V desensitizes TraR to 3OC6HSL without altering its sensitivity to 3OC8HSL.

I58N also has a negative effect on C6HSL sensitivity: $LuxR_{WT}$ was activated both by C6HSL and C8HSL, while $LuxR_{I58N}$ showed no response to C6HSL. LuxRG2E-R67M, a LuxR mutant engineered to be specific for acyl-HSLs lacking a 3-oxo group (e.g., Collins et al., 2006), was originally sensitive to both C6HSL and C8HSL, but when the I58N mutation was introduced into this variant, the mutant receptor selectively lost sensitivity to C6HSL (Fig. S2). Thus, I58N abolishes sensitivity to acyl-HSLs with shorter (C6) side chains in various backgrounds irrespective of the presence or absence of the 3-oxo group. The sensitivity to 3OC10HSL and 3OC12HSL was unaffected by I58N (data not shown).

Y62H confers apparent specificity to acyl-HSLs with shorter side chains

Y62 is highly conserved among LuxR family members (e.g., Whitehead et al., 2001) and is believed to form a hydrogen bond with the ring carbonyl group in acyl-HSLs (e.g., Koch et al., 2005). Previously, Koch et al. (2005) claimed that Y62F abolished the binding of both 3OC6HSL and 3OC8HSL. We assume that Y62H also eliminated the hydrogen bond with the ring carbonyl group, thereby desensitizing LuxR both to 3OC8HSL and 3OC6-HSL.

Y62H dominated our search for 3OC6HSL-specific variants. This is probably because of the nature of OFF-selection (dP selection) used in this work. To survive this selection, the transcriptional switches must be highly stringent (e.g., Tashiro et al., 2011). It is likely that the Y62H mutation was the only accessible molecular solution for LuxR to become completely insensitive to 3OC8HSL.

To recover the activation level (expression level) in the presence of 3OC6HSL, $LuxR_{Y62H}$ was subjected to a second round of mutagenesis and GFP screening for higher fluorescence. We isolated many variants with elevated sensitivity while maintaining the preference for 3OC6HSL over 3OC8HSL (for instance, $LuxR_{mut4}$, Fig. 2D). All four of the variants we sequenced were unique, indicating the existence of multiple solutions for recovering the activation efficiency of $LuxR_{Y62H}$ in its ON state (Table S1). Combinatorial testing of their mutations or additional rounds of mutagenesis and screening should further restore the sensitivity and activation efficiency of 3OC6HSL-specific variants.

A mechanism enabling the rapid evolution of signal specificity

The functional plasticity (or evolvability) of LuxR family proteins has been repeatedly demonstrated either by mutagenesis (e.g., Collins et al., 2005, 2006; Hawkins et al., 2007; Luo et al., 2003) or by the functional comparison of those isolated from closely-related strains (e.g., Chatterjee et al., 2005; Morohoshi et al., 2008).

In previous studies, the signal specificity of the LuxR family has mainly been discussed in terms of altering the pocket shape to fine-tune the interactions. Many of the mutations that have been obtained by directed evolution were also discussed in this context (e.g., Collins et al., 2005, 2006; Hawkins et al., 2007; Luo et al., 2003). However, we were not able to make simple, structure-based explanations of how the mutants isolated in this study operate. To complete the signal response, the LuxR-signal complex must undergo the transition from an inactive to an active state, followed by dimerization, DNA binding, and the recruitment of RNA polymerase (e.g., Fuqua et al., 2001). Many additional features, such as the positive effect of signal binding on the half-life of LuxR *in vivo* (e.g., Manefield et al., 2002) and the localization of LuxR in the membrane in the absence of a ligand (e.g., Kolibachuk and Greenberg, 1993), could affect the apparent behavior of LuxR.

In the directed evolution experiments described in this study, the majority of the specificity changes in LuxR proceeded through altered efficiency in the transition to the active form upon binding of each acyl-HSL, rather than in alterations to the binding affinity for each of the acyl-HSLs. LuxRs are probably capable of binding to many different acyl-HSLs, but only a subset of these acyl-HSLs can trigger their subsequent transformation into the active form. This mechanism is likely the prevailing source of molecular solutions to various other evolutionary or engineering demands. It is reported that LuxR-expressing cells take up many different acyl-HSL molecules irrespective of their EC_{50} values (e.g., Schaefer et al., 1996).

A mechanism of the evolution of signal orthogonality and cross-inhibition

In nature, quorum sensing systems seem to be under diversifying selection (e.g., Ansaldi et al., 2002; Ichihara et al., 2006). The study on Erwinia carotovola (e.g., Chatterjee et al., 2005) revealed that the strain could be categorized into two groups based on their signaling specificity. Here, the Class II signaling molecule 3OC6HSL not only failed to activate Class I receptors, but also inhibited them from responding to their cognate signal 3OC8HSL. Similarly, the Class I signal 3OC8HSL inhibited Class II ExpRs from responding to 3OC6HSL. This evolution of intra-species cross-inhibition was also described for the quorum sensing systems of Chromobacterium (e.g., Morohoshi et al., 2008). However, it is not been clear whether this diversifying selection, if any, drove the evolution of orthogonality and cross-inhibition. Recently, Eldar and his colleagues elegantly demonstrated that the functional divergence, evolution of orthogonality and cross-inhibition of quorum-sensing systems could be beneficial to the population (e.g., Eldar, 2011), and they are indeed rapidly selected and stably maintained in bacterial population by rounds of cheating and immunity from cheating (e.g., Pollak et al., 2016).

While social conflict can serve as an evolutionary driver of inventing and maintaining diversity, orthogonality, and

cross-inhibition, the evolvable nature of LuxRs should also be a great contributor to the rapid evolution of quorum sensing systems. We found that 3OC8HSL-specific LuxR variants ($LuxR_{I58N}$) are inhibited by the presence of 3OC6HSL (Fig. 4). The same applied to LuxRG2E-R67M (e.g., Collins et al., 2006), the previously engineered LuxR variant specific to acyl-HSLs lacking a 3-oxo group (such as C8HSL). In spite that LuxRG2E-R67M is completely insensitive to 3OC6HSL and 3OC8HSL (not shown) (e.g., Collins et al., 2006), its dose-response curve to C8HSL is right-shifted parallel with the co-existence of 3OC8HSL (Fig. S3). Thus, it seems that LuxRs quickly evolve specificity by failing to become activated upon signal binding, rather than failing to bind with the signal. More importantly, with this mechanism, the loss of sensitivity to a cognate signal naturally accompanies the switching of one agonist molecule into an antagonist. If LuxR family proteins frequently undergo specificity switching by this mechanism, it is understandable that we frequently observe the cross-inhibition of closely related quorum-sensing systems in diverging populations of bacteria (e.g., Ansaldi and Dubnau, 2004; Chatterjee et al., 2005; Ji et al., 1997; Morohoshi et al., 2008).

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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