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Directed Evolution of the Quorum-Sensing Regulator EsaR for Increased Signal Sensitivity

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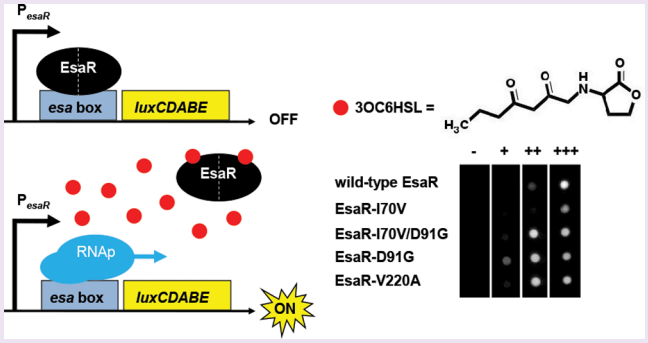
Supporting Information

ABSTRACT: The use of cell−cell communication or "quorum sensing (QS)" elements from Gram-negative Proteobacteria has enabled synthetic biologists to begin engineering systems composed of multiple interacting organisms. However, additional tools are necessary if we are to progress toward synthetic microbial consortia that exhibit more complex, dynamic behaviors. EsaR from *Pantoea stewartii* subsp. *stewartii* is a QS regulator that binds to DNA as an apoprotein and releases the DNA when it binds to its cognate signal molecule, 3-oxohexanoyl-homoserine lactone (3OC6HSL). In the absence of 3OC6HSL, EsaR binds to DNA and can act as either an activator or a repressor of transcription. Gene expression from P_{esaR}, which is repressed by wild-type EsaR, requires 100- to 1000-fold higher concentrations of signal than commonly used QS activators, such as LuxR and LasR. Here we have identified EsaR variants with increased sensitivity to 3OC6HSL using directed evolution and a dual ON/OFF screening strategy. Although we targeted EsaR-dependent derepression of P_{esaR}, our EsaR variants also showed increased 3OC6HSL sensitivity at a second promoter, P_{esaS}, which is activated by EsaR in the absence of 3OC6HSL. Here, the increase in AHL sensitivity led to gene expression being turned off at lower concentrations of 3OC6HSL. Overall, we have increased the signal sensitivity of EsaR more than 70-fold and generated a set of EsaR variants that recognize 3OC6HSL concentrations ranging over 4 orders of magnitude. QS-dependent transcriptional regulators that bind to DNA and are active in the absence of a QS signal represent a new set of tools for engineering cell−cell communication-dependent gene expression.



The biosynthesis of compounds of medical and industrial importance often requires engineering and optimization of complex metabolic pathways. Traditionally, these processes have employed a clonal population of recombinant microbes such as *E. coli* or yeast. There are many limitations of using a single population that could be alleviated or addressed by using a mixture of several organisms. Examples of challenges that may be addressed using synthetic microbial consortia include the metabolic load on individual organisms and the number of exogenous elements that can be contained and optimized in a single cell.[1,2] To fully realize the potential of microbial communities for synthetic biology applications, we must develop engineering strategies to precisely control their behavior in complex environments.

Cell−cell communication is an effective mechanism for regulating cellular behavior in synthetic microbial communities. To engineer cell−cell communication, we take advantage of QS, a mechanism by which bacteria broadcast and respond to changes in their local population density.[3,4] Many Proteobacteria sense their density and coordinate responses via a class of small-molecule signals called acyl-homoserine lactones (AHLs).[5] The canonical example is the *lux* system from marine bacterium *Vibrio fischeri*. LuxI is an AHL synthase,[6] and LuxR is an AHL-dependent transcriptional activator.[7−9] The AHL, 3-oxo-hexanoyl-homoserine lactone (3OC6HSL), is produced inside the cell but can freely diffuse across the cell membrane into the environment.[6,8] Therefore, the AHL concentration is low at a low cell density and increases with the cell density. As the concentration of AHL increases, it is bound by LuxR to form a complex capable of activating gene expression. Pairs of LuxR/LuxI homologues have been identified in many species of Proteobacteria,[10,11] where these QS systems are critical for regulating diverse physiological functions, such as bioluminescence, biofilm formation, and bacterial virulence in many different microbes.[12] Different species of Proteobacteria synthesize and respond to AHLs with acyl chains of varying lengths and modifications.[13−15] These and other types of QS systems allow bacterial populations to act cooperatively to achieve tasks that are not possible by or would not be beneficial to individual cells.[16] Several AHL-based QS modules have also been used in synthetic regulatory systems to mediate cellular communications from single populations to multicellular systems.[17] That is, complex

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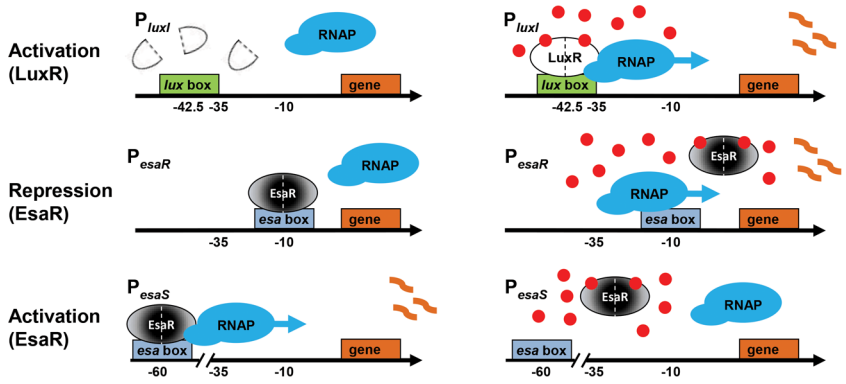

Figure 1. Illustration of AHL-dependent activation and repression of gene expression by LuxR and EsaR. (a) LuxR activates expression at P_{luxI}. (b) EsaR represses transcription at P_{esaR}. (c) EsaR activates transcription at P_{esaS}. For a−c, the left and right panels compare gene expression in the absence and presence of 3OC6HSL (red circles), respectively.

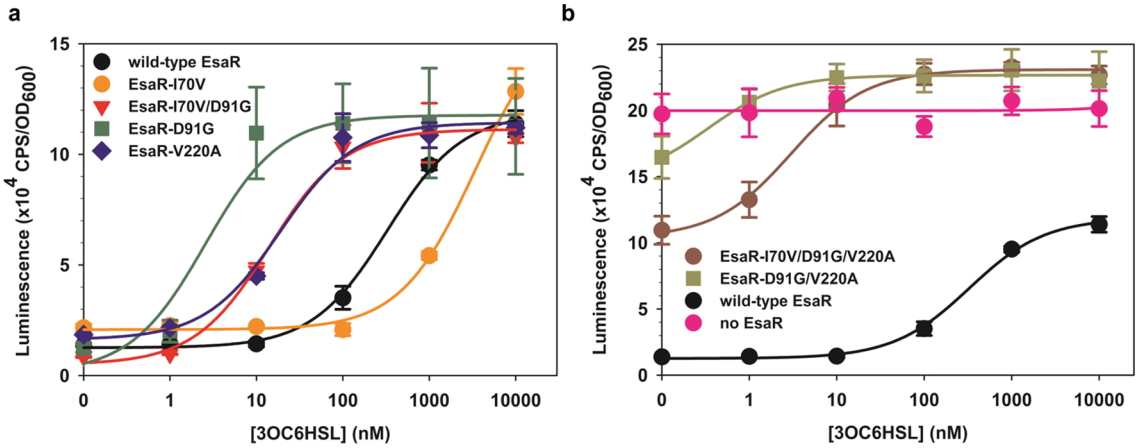

Figure 2. Luminescence assay of 3OC6HSL-dependent derepression of P_{esaR} by evolved EsaR variants. (a) Dose response of wild type and first-generation EsaR variants to 3OC6HSL. (b) Dose response of recombined variants and wild-type EsaR. Promoter activity in the absence of any regulator, shown in pink, indicates that complete derepression of P_{esaR} was observed with the recombined EsaR variants but not with wild type or the first-generation variants. Luminescence data were normalized by OD_{600} to account for subtle differences in cell density following resuspension of cells in bioassay media. Error bars show standard deviations from three independent biological replicates.

regulatory networks including logic gates[18] can be built by manipulating QS systems.

To date, the cell−cell communication modules employed by synthetic biologists have relied on the ability of LuxR and its homologues to activate gene expression upon binding a diffusible AHL signal. In microbes, transcriptional activators function by recruiting RNA polymerase (RNAP) to a promoter sequence and can function by increasing the binding affinity between the promoter and RNAP or by decreasing the energy required for open complex formation (Figure 1a).[19−21] Activators commonly function through interactions with the α- or σ-subunits of RNAP.[20] In contrast, transcriptional repressors simply bind to operator regions within a promoter and inhibit the initiation of transcription (Figure 1b).[19,21]

Transcriptional repressors have been used much more frequently in synthetic circuits than activators. While the placement of an activator-binding site within a promoter is quite rigid for a given activator, the placement of repressor binding sites is more flexible.[22] Repressors can block transcription if they are located between the −35 and −10 sites or downstream of the −10 sites and can even overlap with the −10 or −35 sites.[23] Multiple operator sites can also be added within a single promoter to enable more complex control strategies.[24,25] For example, a synthetic AND gate was recently described that contains binding sites for both the lactose and tetracycline repressors.[24] The synthetic AND gate is analogous to a digital AND logic gate where a high output (gene expression) is only obtained when two inputs (lactose and anhydrotetracycline) are both high. The addition of QS repressors to the list of parts available for synthetic biology would enable the generation of similar hybrid promoters. In addition, network motifs such as negative feedback loops could be constructed using a single regulator. This is in contrast to systems where such a motif is achieved using a QS activator to turn on the expression of a repressor. The use of a QS repressor would both minimize the number of parts required and, by avoiding the additional time required for the transcription and translation of a second regulator, enable the characterization and the use of the more dynamic behaviors expected with feedback motifs.

Placing LuxR's target DNA sequence, the *lux* box, between the −35 and −10 sites or downstream of the −10 site can convert LuxR to a repressor.[26] However, this system has been shown to have low stability and requires the addition of AHL to promote repression. Although they have not been used for synthetic biology previously, there are a small number of LuxR homologues that naturally act as repressors.[27] EsaR is a LuxR homologue from *Pantoea stewartii* subsp. *stewartii* that can

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repress gene expression by binding a promoter, such as P_{esaR}, in the absence of the same AHL as LuxR, 3OC6HSL.[28] In this case, EsaR represses gene expression by binding to its target DNA sequence, the *esa* box, in the absence of 3OC6HSL. In the presence of 3OC6HSL, wild-type EsaR releases the *esa* box, leading to an increase in gene expression (Figures 1b and 2). EsaR has also been shown to activate the expression of a small noncoding RNA from a promoter, P_{esaS}, which is transcribed divergently from P_{esaR} (Figure 1c).[29] Unlike LuxR, however, EsaR activates gene expression in the absence of 3OC6HSL and gene expression is decreased upon 3OC6HSL binding and dissociation of EsaR from the promoter.

Wild-type EsaR requires micromolar concentrations of AHL to induce gene expression from P_{esaR},[28] while QS activators commonly used for synthetic biology exhibit increases in gene expression with nanomolar concentrations.[30] The goal of this work was to generate EsaR variants with increased AHL sensitivity, specifically focusing on EsaR's activity as a repressor and its AHL-dependent derepression of P_{esaR}. Previous work used directed evolution to broaden AHL-binding specificity and increase AHL sensitivity of LuxR.[30] A dual selection system was subsequently used to generate LuxR variants with refocused specificity toward long, straight chain fatty acid containing AHLs.[31] In addition to the generation of novel QS activators, these studies have increased our understanding of the biochemistry of these proteins, which exhibit significant changes in function and stability in response to AHL binding. To date, little understanding of the biochemistry and dynamics of EsaR-dependent gene regulation is available.

We have engineered the AHL-dependent transcriptional regulator, EsaR, to enable its use in synthetic microbial communities. We have used directed evolution and a dual ON/OFF screening strategy, adapted from Collins et al.,[31] to identify a number of EsaR variants that respond to AHL concentrations between 5 and 10,000 nM. The availability of intercellular communication-dependent repressors will enable new circuits and network behaviors not feasible with existing parts (e.g., the transcriptional activator LuxR). Further, these new transcriptional regulators will enable construction of novel engineered density-dependent or multicellular systems for metabolic engineering applications, where the same regulator can be used to either up- or down-regulate gene expression in response to a single QS signal.

RESULTS AND DISCUSSION

ON/OFF Screening Identifies EsaR Variants with Increased AHL Sensitivity As Repressors of P_{esaR}. We used an ON/OFF dual screening approach to identify EsaR variants with increased sensitivity to 3OC6HSL. The system consists of two plasmids, an EsaR expression plasmid and a reporter plasmid where the *lux* operon, *luxCDABE*, is under the control of P_{esaR}. As shown in Figure 2, initial characterization of our two-plasmid system in *E. coli* DH5α showed 3OC6HSL-dependent luminescence. The concentration of 3OC6HSL required to achieve half-maximal luminescence, which we have designated as the apparent dissociation constant ($K_{D(app)}$), was observed to be 210 nM 3OC6HSL for wild-type EsaR (Table 1). We note that the screening and *in vivo* characterization assays described in this work were all conducted at 37 °C.

We used error-prone PCR[32] to generate a library of *esaR* mutants. The library was transformed into competent cells containing the luciferase reporter and screened on LB agar plates in the absence of 3OC6HSL. In the first round of OFF screening, we selected all non-luminescent colonies in order to narrow the library down to only EsaR variants that retain their ability to repress gene expression (i.e., functional repressors). From a library of approximately 25,000 clones we identified 6,500 non-luminescent colonies. Positive clones were cultured in deep-well plates, and a pin stamp tool was used to array the clones on LB agar plates containing 50 nM 3OC6HSL. Under these conditions, luminescence was not observed with wild-type EsaR. In this round of ON screening, luminescence was detected from 191 variants. The luminescence of the 191 clones from our OFF/ON screen was assessed on LB agar plates containing 0, 10, 20, 50, and 100 nM 3OC6HSL. This rescreening step identified 18 clones that exhibited luminescence in response to 50 nM 3OC6HSL. A liquid-phase microtiter plate assay, recloning of the *esaR* mutants into fresh pAC vector, and sequencing led to the identification of three unique EsaR variants, EsaR-I70V/D91G, -V220A, and -D91G, that respond to low nanomolar concentrations of 3OC6HSL.

Table 1. $K_{D(app)}$ Values for Derepression of P_{esaR} by Wild-Type EsaR and the First-Generation EsaR Variants with 3OC6HSL and C6HSL

EsaR type	$[3OC6HSL]_{50}$ (nM) at P_{esaR}[a]	$[C6HSL]_{50}$ (nM) at P_{esaR}[a]
wt EsaR	210 ± 110	ND[b]
EsaR-I70V	3100 ± 670	ND
EsaR-D91G	2.8 ± 0.6	240 ± 180
EsaR-I70V/D91G	15 ± 3	3100 ± 2600
EsaR-V220A	17 ± 2	1400 ± 45

[a] $[3OC6HSL]_{50}$ and $[C6HSL]_{50}$ at P_{esaR} are the concentrations required to reach half-maximal luminescence levels at the P_{esaR} promoter. [b] ND: not detectable.

A liquid-phase bioassay, adapted from Collins et al.,[30] was used to quantitatively characterize AHL-dependent control of gene expression with each EsaR variant. We measured AHL-dependent derepression of P_{esaR} using our luciferase reporter, where luminescence was measured using a microtiter plate reader. The luminescence output was normalized by OD_{600} to account for variation in cell density following the resuspension of the cells into bioassay media. However, no significant differences in growth were observed between strains expressing wild-type EsaR and any of the EsaR variants. Of the three variants identified in our screen, EsaR-D91G responds to the lowest concentration of 3OC6HSL, with a $K_{D(app)}$ of 2.8 nM 3OC6HSL, while the $K_{D(app)}$ values for both EsaR-I70V/D91G and EsaR-V220A were observed to be 15−20 nM 3OC6HSL (Table 1, Figure 2a). While similar minimum and maximum luminescence levels were observed with 0 and 10 μM AHL, respectively, for each EsaR variant, the 3OC6HSL sensitivity of the evolved EsaR variants was significantly increased, as evidenced by the 20- to 100-fold lower $K_{D(app)}$ values relative to wild type (Figure 2a).

Site-Directed Mutagenesis Shows Antagonistic Roles for I70V and D91G Mutations. A glutamic acid to glycine mutation at amino acid 91 was identified in two of our EsaR variants, where D91G on its own conferred the greatest sensitivity to 3OC6HSL and the double mutant with I70V was intermediate between the single mutation and wild-type EsaR. To investigate the roles of the individual mutations, we used site-directed mutagenesis to generate EsaR-I70V. AHL-dependent derepression of P_{esaR} in the presence of EsaR-I70V required even higher concentrations of 3OC6HSL than wild-type EsaR

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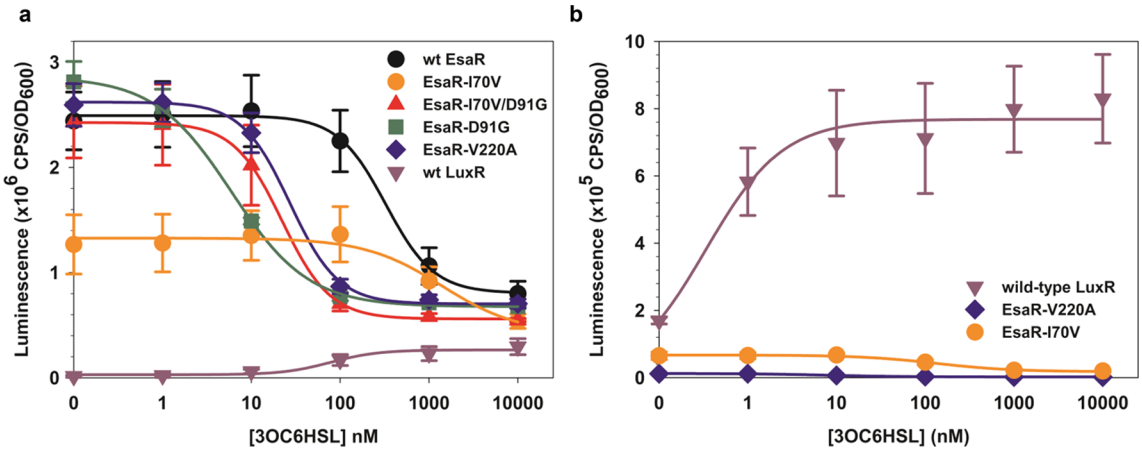

Figure 3. Gene activation assays show differences in promoter specificity and 3OC6HSL sensitivity by EsaR, first-generation EsaR variants, and LuxR. (a) Dose response of wild-type LuxR, wild-type EsaR, and EsaR variants at the P_{esaS} promoter. (b) Dose response of wild-type LuxR and the EsaR variants that exhibited the lowest (EsaR-V220A) and highest (EsaR-I70V) luminescence levels at the P_{luxI} promoter. Luminescence data were normalized by OD_{600} to account for subtle differences in cell density following resuspension of cells in bioassay media. Error bars show standard deviations from three independent biological replicates.

(Figure 2a). As shown in Table 1, 3 μM 3OC6HSL was required for EsaR-I70V to induce half-maximal expression. This value is more than 14-fold higher than the $K_{D(app)}$ for wild-type EsaR. When combined in EsaR-I70V/D91G, these mutations independently exert antagonistic effects on AHL sensitivity resulting in a variant with intermediate sensitivity.

Recombination of First-Generation EsaR Variants. To investigate whether recombination of the first-generation mutations would lead to further increases in 3OC6HSL sensitivity, we constructed two new EsaR variants, EsaR-D91G/V220A and EsaR-I70V/D91G/V220A. While we did observe a subtle increase in 3OC6HSL sensitivity, the recombinant EsaR variants exhibited 8- to 15-fold higher background levels of luminescence relative to wild type and the first-generation EsaR variants (Figure 2b), resulting in a less than 2-fold change in luminescence between ON and OFF states. In comparison, the first-generation EsaR variants exhibit a more than 10-fold increase in luminescence in response to 3OC6HSL. Thus, while recombination of the first-generation mutations led to some increase in sensitivity, these variants have decreased ability to repress gene expression at P_{esaR}. Interestingly, the recombinant EsaR variants also showed significantly higher maximum expression levels relative to our first generation EsaR variants. Luminescence levels observed from P_{esaR} in the absence of EsaR were approximately 2-fold higher than the maximum level observed with EsaR or any of the first-generation EsaR variants. The luminescence observed from the recombined variants is similar to that observed from P_{esaR} in the absence of EsaR (Figure 2b). Together these observations indicate that the AHL-EsaR complexes may still have some affinity for the *esa* box and that there may be a trade-off between minimizing background expression and obtaining maximal expression levels.

EsaR Variants Specificity to AHLs. To assess the AHL-binding specificity of EsaR and our EsaR variants, we measured luminescence in the presence of *N*-butyryl-DL-homoserine lactone (C4HSL), *N*-hexanoyl-DL-homoserine lactone (C6HSL), *N*-octanoyl-DL-homoserine lactone (C8HSL), *N*-decanoyl-DL-homoserine lactone (C10HSL), *N*-dodecanoyl-DL-homoserine lactone (C12HSL), and 3-oxododecanoyl-L-homoserine lactone (3OC12HSL). For wild-type EsaR, AHL-dependent derepression of P_{esaR} was not detected with any non-cognate AHL at concentrations up to 10 μM. However, C6HSL-dependent derepression was observed with each EsaR variant that showed increased sensitivity for 3OC6HSL (Supplementary Figure s1). EsaR-D91G showed the highest sensitivity to C6HSL, with a $K_{D(app)}$ of 240 nM (Table 1). $K_{D(app)}$ values for EsaR-I70V/D91G and -V220A were >1 μM C6HSL. For these two variants, complete derepression was not observed at 10 μM C6HSL (Supplementary Figure s1).

EsaR Variants Show Increased Sensitivity to AHL as Activators of P_{esaS}. To assess the ability of our EsaR variants to activate gene expression, we used a reporter where the *lux* operon is under the control of P_{esaS}. Like their behavior as repressors, the EsaR variants showed increased AHL sensitivity when activating gene expression (Figure 3a, Supplementary Table s1). However, in this case gene expression was turned OFF, instead of ON, by the addition of 3OC6HSL. The fully induced and basal levels of expression observed with 0 and 10 μM AHL, respectively, were the same for wild type and our evolved EsaR variants. However, EsaR-I70V showed a lower level of gene activation in the absence of 3OC6HSL. To assess crosstalk between the *esa* and *lux* promoters, we examined the ability of wild-type LuxR to activate expression from P_{esaS}. As shown in Figure 3a, LuxR does activate gene expression from P_{esaS} in the presence of 3OC6HSL. However, the level of expression is approximately 10-fold less than was observed with EsaR. Further, the minimum level of expression observed with EsaR in the presence of 10 μM 3OC6HSL was 2-fold higher than the maximum level observed with LuxR. Basal expression from P_{esaS} without any QS regulator was similar to that observed with wild-type LuxR in the absence of AHL (data not shown).

Previous studies have shown that EsaR can function as an activator LuxR's cognate promoter (P_{luxI}) from *Vibrio fischeri*.[29,33] To assess the ability of our EsaR variants to activate gene expression from P_{luxI}, we replaced the P_{esaS} promoter with P_{luxI} and repeated the activation assays. Figure 3b compares AHL-dependent gene activation by wild-type LuxR to that of the EsaR variants with the highest (EsaR-I70V) and lowest (EsaR-V220A) ability to activate gene expression at P_{luxI}. In addition, the basal level of expression observed with

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LuxR in the absence of AHL was 3-fold higher than the maximum level observed with EsaR-I70V. Basal expression from P_{luxI} in the absence of regulator was similar to that observed with EsaR and 10 μM 3OC6HSL (data not shown).

EsaR Homology Model. Biochemical studies of LuxR homologues are notoriously challenging due, in many cases, to AHL-dependent stability.[34] While one of the key differences between EsaR and its activator homologues is the fact that it must be stable and bound to DNA in the absence of AHL, purifying this protein has also been challenging.[35] For our part, we generated N- and C-terminal fusions of c-myc and 6x-His tags and found, in each case, that EsaR function was drastically reduced, if not abolished (data not shown). As such, we developed a homology model to illustrate the possible locations of the mutations and to highlight elements that may be responsible for the mechanistic differences between AHL-dependent activators and repressors. We chose TraR as the template for building an EsaR homology model. TraR was the first LuxR homologue to be crystallized (PDB code: 1l3l).[36] In the homology model, the TraR signal molecule, 3-oxooctanoyl-L-homoserine lactone 3OC8HSL, was replaced with 3OC6HSL, and energy minimization on the side chains surrounding 3OC6HSL was conducted to address any collisions resulting from the signal molecule replacement (Figure 4). The AHL-binding pocket in TraR has been

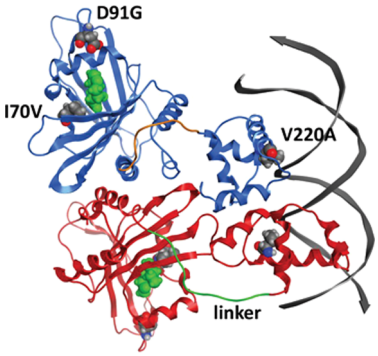

Figure 4. EsaR homology model highlighting mutations affecting AHL sensitivity. The double-stranded DNA to which EsaR binds is shown in dark gray. The EsaR dimer consists of two peptides shown in red and blue. The anti-parallel β-sheets form a pocket with two strands of α-helices for 3OC6HSL binding. The molecule of 3OC6HSL is shown in space-filling green. Three residues, I70V, D91G, and V220A, are shown in space-filling molecule-specific colors.

observed to contain mostly aromatic and hydrophobic residues.[37] Residues with similar properties are present in the AHL-binding pocket of EsaR. The most prominent differences between the EsaR and TraR sequence are the length of the linker between the N- and C-terminal domains as well as the additional residues at the C-terminus (Supplementary Figure s2).[38] The extended linker in EsaR, highlighted in Figure 4, contains nine additional amino acids. This linker region is often found in the family of QS regulators that, like EsaR, are bound to DNA in the absence of AHL.

From the homology model, the I70V and D91G mutations are located in the AHL-binding domain. However, these residues do not make direct contact with the AHL. The I70V mutation aligns with a valine residue at position 73 in TraR. In TraR, V73 is adjacent to two highly conserved residues, D70 and V72, that participate in AHL binding.[36,39] Further, the presence of an isoleucine or valine at this position is highly conserved between homologues and may indicate that this mutation has been used in natural systems to tune AHL sensitivity.[40] The D91G mutation is at the N-terminus of an α-helix that has been shown to include residues involved in AHL binding.[36] Residues aligning with D91 in EsaR homologues are predominantly charged. The V220A mutation is located within the DNA-binding domain. Alanine scanning mutagenesis of TraR showed that a mutation at this position (M213A) resulted in a 50% decrease in DNA-binding affinity,[41] indicating that the observed increase in AHL sensitivity of EsaR-V220A may be due to a decrease in DNA-binding affinity. Interestingly, a similar set of alanine scanning mutagenesis experiments in LuxR showed unappreciable changes in the ability of the analogous variant Q222A to bind to the *lux* box and activate gene expression.[42] The finding that the mutations we have identified may affect AHL-binding affinity or DNA-binding affinity was not unexpected, as either an increase in the affinity for AHL or a decrease in affinity for the *esa* box could cause the observed differences in $K_{D(app)}$ values observed in our variants. However, changes could also occur from other factors, such as conformational changes or changes in protein expression or stability. Biochemical studies on other LuxR homologues, such as LuxR,[42,43] TraR,[37,39,44] QscR,[45] and CviR,[46] have shown that mutations altering signal specificity, DNA binding affinity, and protein stability can all affect gene activation. Future biochemical and structural studies of EsaR and our EsaR variants will undoubtedly provide additional insights into the mechanisms by which the mutations identified here modulate protein function. An EsaR-fusion protein, His_6-maltose binding protein-glycine linker (Gly5)-EsaR (HMGE), was recently developed that yields full-length EsaR following cleavage by a TEV protease.[35] Although protease cleavage was observed to be incomplete, this system did enable the *in vitro* characterization of 3OC6HSL-induced conformational changes to EsaR. Future studies incorporating our mutations into HMGE may lead to insights into the biochemical mechanisms underlying EsaR function and the observed changes in AHL sensitivity.

Conclusions. This work has led to the generation of a set of quorum-sensing dependent regulators with increased 3OC6HSL sensitivity that can act as either activators or repressors of transcription. A set of mutations that modulate AHL sensitivity has been identified and has provided some insight into the biochemistry of this protein. The addition of these tools to the toolbox of QS regulatory systems may enable the generation of new systems that take advantage of EsaR's behavior as a simple cell−cell communication-dependent DNA-binding module that can be used to make hybrid promoters or novel regulatory networks. On its own EsaR can enable both repression at P_{esaR} and activation at P_{esaS}. The opposite responses to 3OC6HSL would allow some genes to be up-regulated while others are down-regulated in a single cell simply by altering the promoter. EsaR variants with increased ability to activate gene expression may be of particular interest and could be targeted with our dual screening strategy. Further, we anticipate that the variants identified in this work will serve as starting materials for the generation of new EsaR variants that recognize different AHLs and enable new lines of cell−cell communication.

METHODS

Plasmid Construction. We constructed a two-plasmid system consisting of the EsaR expression vector, pAC-EsaR, and the reporter plasmid, pP_{esaR}*lux*. pAC-EsaR is an expression plasmid where a *lac*

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promoter, the *esaR* gene, and a transcriptional terminator were cloned between the *Xba*I and *Sal*I sites in pACYC184. The *esaR* gene was PCR-amplified from pTDM6[28] and is flanked by *Kpn*I and *BamH*I restriction sites in pAC-EsaR. In generating pAC-EsaR, a mutation encoding a phenylalanine to valine substitution at the second amino acid introduced during construction of pTDM6 was repaired, such that our parent gene is wild-type *esaR*. P_{esaR} was PCR-amplified from pTDM7[28] using the primers 5′-P_{esaR}-*Xho*I and 3′-P_{esaR}-*Bam*HI and ligated into the *Xho*I and *Bam*HI digested pCS26[47] backbone to generate pCS-P_{esaR}*lux*. Similarly, the P_{luxI} was PCR-amplified from p*luxGFPuv*[30] using 5′-P_{luxI}-*Xho*I and 3′-P_{luxI}-*Bam*HI and ligated into *Xho*I-and-*Bam*HI-digested pCS26. The P_{esaS} was PCR-amplified from pTDM7 using 5′-P_{esaS}-*Xho*I and 3′-P_{esaS}-*Bam*HI and ligated into *Xho*I-and-*Bam*HI-digested pCS26 to generate pCS-P_{esaS}*lux*. Standard PCR-based site directed mutagenesis methods were used to generate both the *esaR* variants with isolated mutations and the recombinant variants. Please see Supporting Information for details in used strain, plasmids, and primers (Supplementary Tables s2 and s3).

Library Construction and Dual ON/OFF Screening. Error-prone PCR reactions were performed using *Taq* DNA polymerase with 5.5 μM $MgCl_2$ and 100 μM $MnCl_2$ to increase the mutation rate as described.[32] The *esaR* gene was PCR-amplified from pAC-EsaR using primers 5′-*esaR-Kpn*I and 3′-*esaR-Bam*HI. The library was constructed by ligating the error-prone PCR-amplified *esaR* genes with *Kpn*I and *Bam*HI digested pAC-EsaR. For the first round of screening (OFF), the ligation mixture was transformed into chemically competent DH5α cells harboring pCS-P_{esaR}*lux* and plated onto LB agar without AHL. The plates were incubated at 37 °C for 24 h prior to screening. To identify clones of interest, the agar plates were screened using a ChemiImager 4400 (Alpha Innotech) and visually inspected to identify non-luminescent colonies. For the second round of screening (ON) the clones of interest from the first round grown overnight in a 96-well plate and then stamped onto LB agar plates containing 50 nM 3OC6HSL using a pin replicator. In this round, only luminescent colonies were isolated. Positive clones from the ON screen were grown overnight in LB and stamped onto plates containing a range of 3OC6HSL concentrations (0, 10, 20, 50, and 100 nM). A 24-h assay utilizing black 96-well microtiter plates with optically clear bottoms was performed to rapidly characterize the EsaR variants identified from the agar plate screening. Overnight cultures were diluted 600-fold into 150 μL of LB medium containing appropriate antibiotics and titrated 3OC6HSL. A layer of mineral oil was added to each well to avoid evaporation. The 96-well microtiter plate was then incubated in Envision multilabel plate reader (Perkin-Elmer) at 37 °C, and OD_{600} and luminescence were measured every 30 min for 24 h. All positive variants identified were recloned into fresh vector backbone and strains to eliminate secondary site-effects.

Quantitative Characterization of EsaR. The assay we performed to measure EsaR-mediated gene expression was adapted from a light-based bioassay protocol.[30] Single colonies were picked to grow cells in LB containing appropriate antibiotics overnight. The overnight cultures were diluted 100-fold into 60 mL of LB containing 5 mM potassium phosphate buffer, pH 6.5, and the appropriate antibiotics and then inoculated in a 37 °C shaker until an OD_{600} measurement of 0.3−0.4. The cell cultures were then centrifuged at 6000*g* for 10 min at RT. The cell pellets were resuspended to an OD_{600} of 0.3 in bioassay medium (0.05% w/v tryptone, 0.03% v/v glycerol, 100 mM sodium chloride, 50 mM magnesium sulfate, and 5 mM potassium phosphate buffer, pH 6.5). The 2 mL of resuspended cells was combined with 0.5 mL of bioassay medium containing AHL in a 48-well plate. AHL concentrations between 0 and 10,000 nM for 3OC6HSL, 3OC12HSL, C4HSL, C6HSL, C8HSL, C10HSL, and C12HSL were assayed. The 48-well plates were then incubated at 37 °C with shaking for 2 h. Thereafter, we transferred 200 μL from each well to a black 96-well microplate with an optically clear bottom. Luminescence and cell densities (600 nm) were measured using an Envision multilabel plate reader (Perkin-Elmer). The luminescence output was normalized by optical density at 600 nm. Three biological replicates of each bioassay condition were conducted, and the luminescence values were averaged. The transfer curves were generated using Sigmaplot. Curve-fitting was conducted using the Hill equation ($Y = Y_0 + [S]^n/(K_m + [S]^n)$, n = Hill coefficient) where the Hill coefficient was set to 1.

Homology Model Construction. A homology-based molecular model for EsaR was created using the Molecular Operating Environment software (MOE 2010.10; Chemical Computing Group). The template was the high-resolution X-ray crystal structure of TraR dimer with bound pheromone 3OC8HSL and double-stranded DNA ligand[36] (PDB code: 1l3l). Briefly, EsaR was aligned to TraR using the BLOSUM62 substitution matrix and no end gap penalty, resulting in 39% sequence identity with four insertions and three deletions. The alignment was modified manually to consolidate and place insertions/deletions (gaps) outside of secondary structure elements. The resulting three loop regions (TraR/EsaR: 42I-47I/35 V-43V, 86S-112S/82D-124S, 160S-176P/172N-182S) were modeled using MOE's automated database search, side chain rotamer search, and energy minimization procedure. The Amber99 force field[48] was used, with a reaction field implicit solvation model for electrostatics.[49] Ten models were generated, and each was evaluated for good stereochemistry, backbone dihedral angles, and side-chain rotamers. In what was judged the best model, the unaligned C-terminal region of EsaR was modeled as an additional β-strand. In this model the natural cognate inducer, 3OC6HSL, was then used to replace 3OC8HSL, and the side chains surrounding the inducer were energy minimized to resolve collisions resulting from the inducer replacement.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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