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Quorum Sensing-Modulated AND-Gate Promoters Control Gene Expression in Response to a Combination of Endogenous and Exogenous Signals

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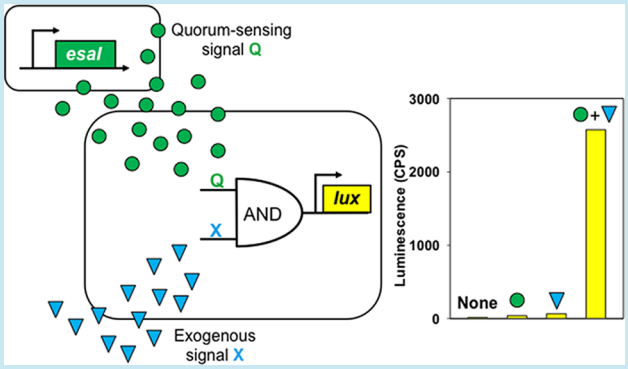
Supporting Information

ABSTRACT: We have constructed and characterized two synthetic AND-gate promoters that require both a quorum-sensing (QS) signal and an exogenously added inducer to turn on gene expression. The engineered promoters, LEE and TTE, contain binding sites for the QS-dependent repressor, EsaR, and either LacI or TetR, and they are induced by an acyl-homoserine lactone (AHL) signal and IPTG or aTc. Although repression of both LEE and TTE by wild-type EsaR was observed, induction of gene expression at physiologically relevant concentrations of AHL required the use of an EsaR variant with higher signal sensitivity. Gene expression from both LEE and TTE was shown to require both signal molecules, and gene expression above background levels was not observed with either signal alone. We added endogenous production of AHL to evaluate the ability of the promoters to function in a QS-dependent manner and observed that gene expression increased as a function of cell density only in the presence of exogenously added IPTG or aTc. Cell−cell communication-dependent AND-gate behaviors were demonstrated using an agar plate assay, where cells containing the engineered promoters were shown to respond to AHL produced by a second *E. coli* strain only in the presence of exogenously added IPTG or aTc. The promoters described in this work demonstrate that EsaR and its target DNA sequence can be used to engineer new promoters to respond to cell density or cell−cell communication. Further, the AND-gate promoters described here may serve as a template for new regulatory systems that integrate QS and the presence of key metabolites or other environmental cues to enable dynamic changes in gene expression for metabolic engineering applications.



KEYWORDS: *Quorum sensing, cell−cell communication, logic gate, EsaR, AND gate, autoinduction*

Cells are able to adapt and survive in continuously changing conditions by adjusting phenotypic behaviors in response to a range of environmental stimuli and cellular signals. This process often involves the integration of multiple extra- and intracellular signals and can lead to changes in gene expression, protein function, metabolism, and even cell−cell communication.[1−5] Natural promoters that respond to multiple signals often include binding sites for multiple transcription factors. For example, the wild-type *lac* promoter is controlled both by LacI and cAMP receptor protein (CRP).[6] Early success in building synthetic devices, such as the genetic toggle switch[7] and repressillator,[8] utilized well-characterized inducible systems (*lac*, *tet*, and *ara*), to manipulate regulatory behaviors in *E. coli*. Components from these inducible systems, specifically transcription factors and their target DNA operator sites, have since been used to build synthetic promoters that respond to multiple inputs and often perform logic-gate functions.[9−11]

In microbial systems, the design of promoters that respond to multiple inputs has most often included placing operators upstream of the −35 site, between the −35 and −10 sites, and/or downstream of the −10 site in σ^{70}-dependent promoters.[9,10,12,13] Ramalingam et al. used such an approach in their design of AND-gate promoters that respond to isopropyl-β-D-1-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc) by combining components from the *tet* and *lac* regulatory systems.[9] The AND-gate promoters are analogous

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to a digital AND logic gate where a high output is only obtained when two inputs are both high (Figure 1a). Each of their promoters contained three operator sites, two *lacO* and one *tetO* (TLL, LTL, LLT), or two *tetO* and one *lacO* (LTT, TLT, TTL).[9] While they found that the promoters that included two *lacO* sites did not show an increase in gene expression even at the highest concentrations of the two inducers, the three promoters that included two *tetO* sites and one *lacO* site showed AND-gate behaviors in the presence of aTc and IPTG.[9] They also observed that leakiness in the presence of one of the two inducers and maximum expression levels varied among LTT, TLT, and TTL.[10] Similar AND gates have been described that incorporate other regulatory components or additional inputs.[10,14−16]

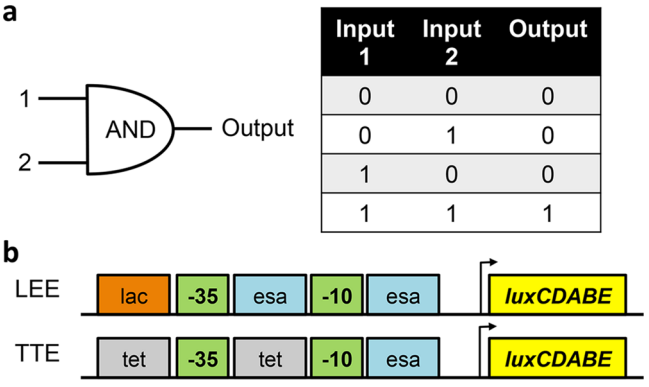

Figure 1. Illustration of the AND logic gate and EsaR-dependent AND-gate promoters. (a) Illustration of a two-input AND gate and its truth table. (b) The LEE and TTE AND-gate promoters contain three operator sites each, one upstream of the −35 site, one between the −35 site and the −10 site, and one downstream of the −10 site. LEE contains one *lacO* site (lac, orange) and two *esa* boxes (esa, blue). TTE contains two *tetO* sites (tet, gray) and one *esa* box. Each promoter was cloned upstream of the *lux* operon.

Although including multiple operators in a single promoter has been used to successfully engineer genetic devices enabling AND-gate-like behaviors, gene expression in these systems is turned on when cells recognize two exogenously added signals. In many situations, however, it may be useful for gene expression to be controlled by a combination of internal and external signals, where the exogenous addition of an inducer would only lead to gene expression when a cell is in a particular physiological state, such as growth phase, population density, or intracellular availability of a metabolite. Here, we focus on building and characterizing genetic AND-gates that respond to both an exogenous signal molecule and an endogenous cell−cell communication signal.

Quorum-sensing (QS) systems allow microbes to respond to changes in local population density via the production and sensing of small molecules, such that the local concentration of the molecule correlates to population density.[17,18] QS components have been used extensively by synthetic biologists to enable cell−cell communication and density-dependent changes in gene expression.[19−23] The system employed most frequently is the *lux* system from *Vibrio fischeri*, which includes the transcription factor LuxR, an acyl-homoserine lactone (AHL) synthase, LuxI, and the QS signal, 3-oxo-hexanoyl-homoserine lactone (3OC6HSL).[24−26] LuxR activates gene expression at the native P_{luxI} promoter by binding to the *lux* box and recruiting RNA polymerase (RNAP) to the promoter.[27] A promoter that exhibits LuxR- and LacI-dependent AND-gate behavior was constructed by placing a *lacO* downstream of the −10 site in P_{luxI}.[16] Ayukawa et al., used a similar approach and showed that the addition of two *lacO* sites downstream of a weak LuxR-dependent promoter, pluxpR, led to a promoter that could only be induced by 3OC6HSL in the absence of LacI.[15] In both of these cases, a promoter that is activated by LuxR was used as the starting point for the generation of the engineered promoters.

Further improvements and the generation of additional multi-input promoters using LuxR are likely to be limited by the fact that the *lux* box must be positioned upstream of the −35 site for LuxR-dependent activation to occur. One alternative approach to enable cells to respond to a combination of inputs is to use tandem promoters, where two promoters are placed sequentially and an mRNA coding for a target protein is generated if either promoter is active.[22] Cells that exhibit OR- or NOR-gate behaviors in response to 3OC6HSL, or an acyl-homoserine lactone (AHL) signal recognized by another LuxR-type activator, and a second exogenous inducer molecule were generated using tandem promoters that consist of P_{luxI} and a second inducible promoter controlled by TetR, LacI, or AraC. A second approach is to use a QS-dependent transcription factor that can behave as a repressor, similar to LacI or TetR, where DNA binding is simply modulated by signal binding.[28,29] Although LuxR has been observed to act as a repressor in the presence of 3OC6HSL when the *lux* box is placed between the −10 and −35 sites or downstream of the −10 site, high basal gene expression levels were observed,[30] indicating that relatively weak binding of LuxR to the *lux box* renders it a poor repressor. Further, Cox et al. developed and characterized a library of combinatorial promoters and observed that LuxR functioned as an activator when the *lux* box was located upstream of the −35 site, while promoters with *lux* boxes between the −10 and −35 sites and/or downstream of the −10 site were neither activated or repressed by the presence of LuxR and 3OC6HSL.[10] To avoid the limitations of the *lux* system, we targeted a QS system where the transcriptional regulator can behave as a repressor. The *esa* QS system from *Pantoea stewartii* includes the transcriptional regulator, EsaR, which recognizes the same QS signal as LuxR, 3OC6HSL, but can behave as a repressor.[31] Unlike LuxR, 3OC6HSL binding causes EsaR to leave its target DNA sequence, the *esa* box.[32] For example, EsaR acts as a repressor at P_{esaR}, where the *esa* box overlaps the −10 site, and gene expression is induced following 3OC6HSL binding.[31,32] While EsaR behaves more similar to LacI and TetR than LuxR, and may be more flexible for the design of multi-input promoters, it is not without limitations. EsaR has been shown to act as a weak activator when the *esa* box is placed upstream of the −35 site.[33−35] Therefore, the *esa* box should be positioned downstream of the −35 site to enable EsaR-dependent repression in engineered promoters.

We have previously described the generation of a set of EsaR variants with increased 3OC6HSL sensitivity via directed evolution,[36] and the generation of a set of EsaR-dependent promoters with a range of expression levels via the addition of a second *esa* box to the P_{esaR} promoter.[35] To assess whether components from the *esa* system, specifically EsaR and the *esa* box, can be used in the design of multiple input promoters that respond to cell−cell communication and an exogenously added signal, we designed two synthetic AND-gate promoters that contain the *esa* box and either *tetO* or *lacO*. Here, we describe

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the characterization of these promoters and show that the desired AND-gate behavior is observed when 3OC6HSL and either IPTG or aTc are added exogenously. Further, we demonstrate that endogenous 3OC6HSL production enables QS-dependent gene expression only in the presence of a second exogenous signal. Finally, we demonstrate that the promoters can enable cell−cell communication-dependent AND-gate behaviors in the presence of a second *E. coli* strain producing 3OC6HSL.

RESULTS

AND-Gate Promoter Design and Characterization. To investigate the behaviors of a bacterial expression system that combines the regulatory ability of EsaR with either TetR or LacI, we designed two-input AND-gate promoters that include the *esa* box and either *lacO* or *tetO* sites (Figure 1b and Figure S1, Supporting Information). Our design was based on the synthetic AND-gate promoters described by Ramalingam et al., which contained two *tetO* sites and one *lacO* site and exhibited the desired behaviors in the presence of IPTG and aTc.[9] Because the *esa* box cannot be placed upstream of the −35 site, we replaced the *tet* operators in LTT with two *esa* boxes to generate LEE and the *lac* operator in TTL with an *esa* box to construct TTE (Figure 1b and Figure S1, Supporting Information). The two AND-gate promoters, LEE and TTE, were generated by DNA synthesis and cloned upstream of the luciferase operon, *luxCDABE*, in a plasmid containing an SC101 origin of replication and a kanamycin resistance gene to generate pLEE and pTTE. To investigate whether our AND-gate promoters were active, luminescence from LEE and TTE was measured in the absence of EsaR, TetR, and LacI repressors in *E. coli* DH5α. We observed a 20-fold increase in luminescence from LEE and a 130-fold increase in luminescence from TTE relative to background levels observed from cells containing a promoterless vector control, indicating that both LEE and TTE are functional promoters (Figure S2, Supporting Information).

pLEE, and pAC-σ^{70}-EsaR, which constitutively expresses wild-type *esaR* from a σ^{70}-dependent promoter, were introduced into a strain of *E. coli*, JM109, that constitutively expresses LacI. For clarity, this promoter/EsaR combination is designated LEE/EsaR. We conducted liquid-phase assays by titrating IPTG from 0 to 1 mM and 3OC6HSL from 0 to 10 μM and measured luminescence with each combination of the two inducer concentrations. As expected, only background levels of luminescence comparable to the promoterless vector control were observed in the absence of IPTG and 3OC6HSL (Figure 2a). However, an increase in luminescence was not observed in the presence of the highest concentrations of both IPTG and 3OC6HSL (Figure 2a). To assess whether binding of wild-type EsaR at LEE was too tight within the range of 3OC6HSL concentrations tested, we replaced it with two EsaR variants, EsaR-V220A and -D91G, which are 12- and 70-fold more sensitive to 3OC6HSL than wild type,[36] to generate LEE/EsaR-V220A and LEE/EsaR-D91G. In the absence of either IPTG or 3OC6HSL alone, only background luminescence was observed from LEE/EsaR-V220A and LEE/EsaR-D91G (Figure 2b and c). Luminescence above background from LEE/EsaR-V220A was observed with 0.01 mM IPTG and 1 μM 3OC6HSL (Figure 2b), while LEE/EsaR-D91G required the same concentration of IPTG but only 100 nM 3OC6HSL (Figure 2c). Further increases in luminescence were observed as the concentrations of each inducer were increased. With

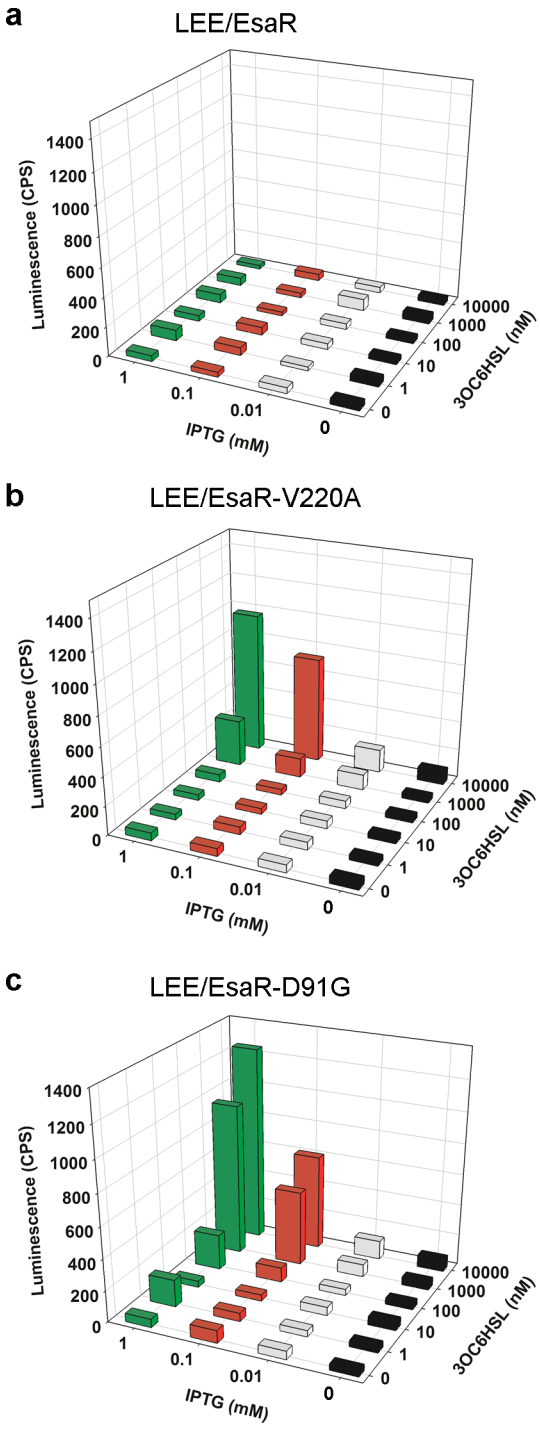

Figure 2. Gene expression from LEE shows 3OC6HSL and IPTG-dependent AND-gate behavior. Luminescence levels from LEE with (a) wild-type EsaR, (b) EsaR-V220A, and (c) EsaR-D91G were determined in the presence of 24 combinations of IPTG (0, 0.01, 0.1, and 1 mM) and 3OC6HSL (0, 1, 10, 100, 1000, and 10000 nM) concentrations. The *E. coli* strain used in these assays, JM109, constitutively expresses LacI. Luminescence values show the mean from three independent biological replicates.

1 μM 3OC6HSL and 0.1 or 1 mM IPTG, luminescence from LEE/EsaR-D91G was 3- to 4-fold higher than LEE/EsaR-V220A (Figure 2b and c), indicating that LEE/EsaR-D91G is more sensitive to 3OC6HSL than LEE/EsaR-V220A. The maximum level of luminescence from LEE/EsaR-D91G was

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also 20% higher than LEE/EsaR-V220A in the presence of 1 mM IPTG and 10 μM 3OC6HSL (Figure 2b and c). At the highest concentrations of IPTG (1 mM) and 3OC6HSL (10 μM) tested, 14- and 28-fold increases in gene expression were observed from LEE/EsaR-V220A and -D91G relative to background.

pTTE was introduced into *E. coli* DH5α-PRO, which constitutively produces TetR, together with pAC-σ^{70}-EsaR, pAC-σ^{70}-EsaR-D91G, or pAC-σ^{70}-EsaR-V220A (TTE/EsaR, TTE/EsaR-D91G, and TTE/EsaR-V220A). We conducted liquid-phase assays titrating aTc from 0 to 200 ng/mL and 3OC6HSL from 0 to 10 μM and measured luminescence from cells in the presence of each combination of the two inducer concentrations. Similar to LEE, only background luminescence was observed from TTE/EsaR in the absence of any inducer. Background luminescence was also observed in the presence of both 200 ng/mL aTc and 10 μM 3OC6HSL (Figure 3a). When wild-type EsaR was replaced with EsaR-V220A or -D91G, luminescence above background was observed with 10 ng/mL aTc and 100 nM 3OC6HSL (Figure 3b and c). Further increases in luminescence from TTE/EsaR-D91G and TTE/EsaR-V220A were observed as the concentration of either aTc or 3OC6HSL was increased. The maximum level of luminescence from TTE was observed with 200 ng/mL aTc and 10 μM 3OC6HSL, where a 100- to 200-fold change in gene expression was observed from both TTE/EsaR-D91G and TTE/EsaR-V220A (Figure 3b and c). Unlike LEE, 3OC6HSL sensitivity and the levels of gene expression from TTE were similar with EsaR-V220A and EsaR-D91G.

Replacing the two *tet* operators in LTT, which exhibited leaky gene expression at high concentrations of IPTG in the absence of aTc, with two *esa* boxes in LEE led to a promoter with minimal leaky gene expression. Although the reported DNA-binding affinities for both EsaR and TetR for their respective DNA targets are approximately 10^{-8} M,[32,37] this suggests that EsaR may have a higher affinity for the two *esa* boxes in LEE than TetR does for the two *tetO* in LTT. The apparent increase in affinity displayed by EsaR may be due to differences in how the two repressors are affected by crowding of the transcription factors on the DNA due to the proximity of the binding sites, and other properties of the DNA surrounding the operator sites.

A higher concentration of 3OC6HSL was required to induce gene expression from LEE relative to TTE when the highest concentrations of IPTG and aTc were present (Figure 2b and c, Figure 3b and c). In addition, gene expression from LEE/EsaR-D91G under these conditions required lower concentrations of 3OC6HSL than LEE/EsaR-V220A. Similar concentrations of 3OC6HSL were required for both TTE/EsaR-V220A and TTE/EsaR-D91G (Figure 2b and c, Figure 3b and c). These results indicate that the second *esa* box increases the overall affinity of EsaR for the promoter and suggest that the number *esa* boxes can be used to tune 3OC6HSL sensitivity. This is consistent with our previous study of P_{esaR}, where we observed that adding a second *esa* box between the −35 and −10 sites or downstream of the −10 site leads to changes in both gene expression levels and 3OC6HSL sensitivity.[35]

Quorum Sensing-Dependent Gene Expression of the *esa*-Based AND Gates. To explore whether our new AND-gate promoters are able to integrate an exogenous signal and an endogenous signal, we added constitutive expression of a 3OC6HSL synthase, EsaI, to the system. Specifically, *esaI* was cloned downstream of *esaR-V220A* and *esaR-D91G* to generate

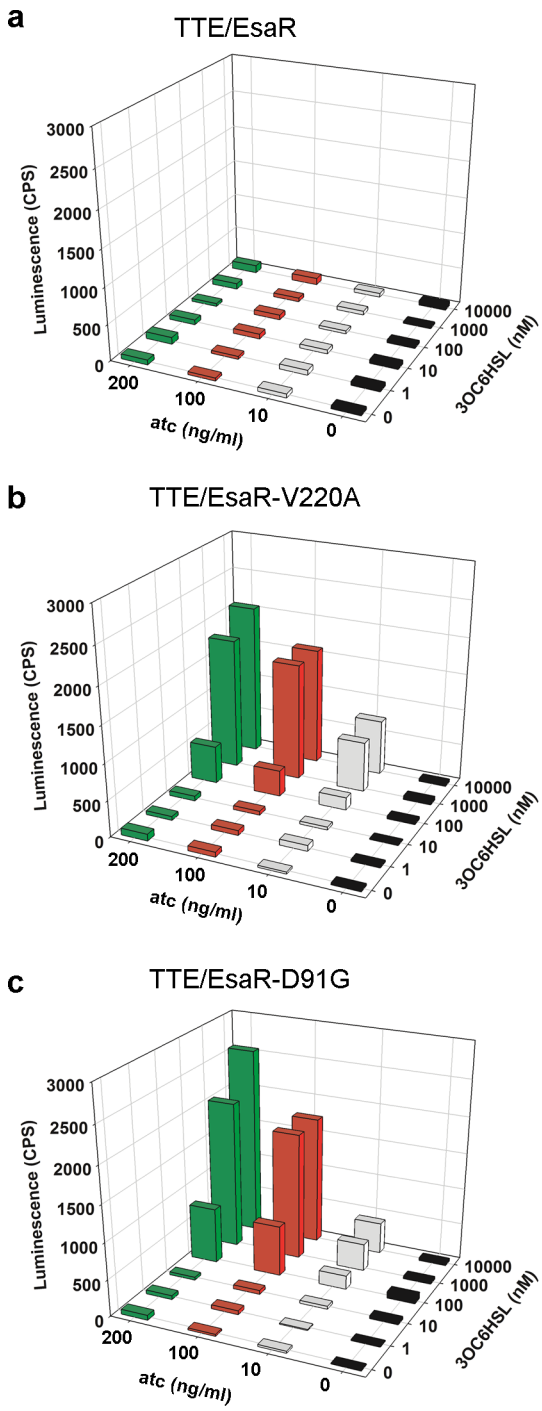

Figure 3. Gene expression from TTE shows 3OC6HSL and aTc-dependent AND-gate behavior. Luminescence levels from LEE with (a) wild-type EsaR, (b) EsaR-V220A, and (c) EsaR-D91G were determined in the presence of 24 combinations of aTc (0, 10, 100, and 200 ng/mL) and 3OC6HSL (0, 1, 10, 100, 1000, and 10000 nM) concentrations. The *E. coli* strain used in these assays, DH5α-PRO, constitutively expresses TetR. Luminescence values show the mean from three independent biological replicates.

pAC-σ^{70}-EsaR-V220A-EsaI and pAC-σ^{70}-EsaR-D91G-EsaI, where both genes are constitutively expressed. We conducted assays in flasks with shaking and monitored cell growth (OD_{600}) and luminescence every 2 h from inoculation until the cultures reached stationary phase. We anticipated that the concentration of 3OC6HSL in cultures would increase as the

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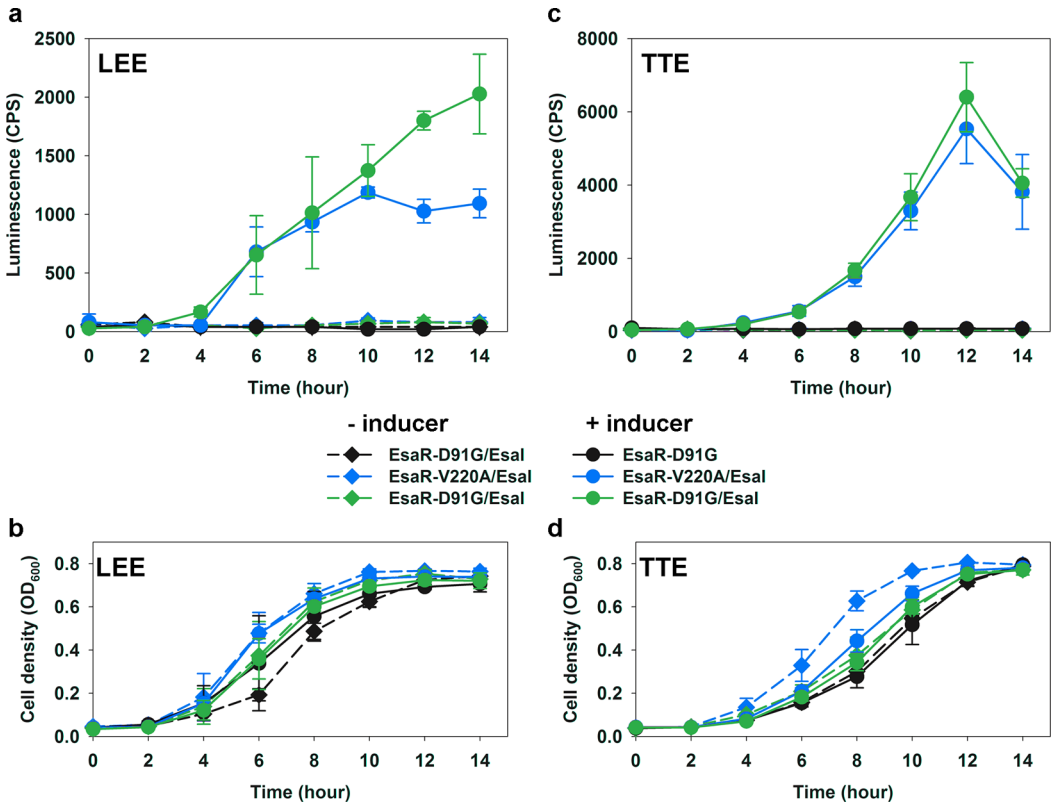

Figure 4. Endogenous 3OC6HSL production enables quorum sensing-dependent AND-gate behavior from LEE and TTE. (a) Luminescence and (b) cell density of two LEE strains that produce endogenous 3OC6HSL (LEE/EsaR-D91G/EsaI, LEE/EsaR-V220A/EsaI) and one strain that does not produce 3OC6HSL (LEE/EsaR-D91G) were measured without IPTG (-inducer) and in the presence of 1 mM IPTG (+inducer). (c) Luminescence and (d) cell density of two TTE strains that produce endogenous 3OC6HSL (TTE/EsaR-D91G/EsaI, TTE/EsaR-V220A/EsaI) and one strain that does not produce 3OC6HSL (TTE/EsaR-D91G) were measured without aTc (−inducer) and in the presence of 100 ng/mL aTc (+inducer). Error bars show standard deviations from three independent biological replicates.

number of EsaI-expressing cells increases during cell growth and that this would result in QS-dependent gene expression only if the exogenous signal molecule, IPTG or aTc, was also added to the cultures.

To determine background levels of gene expression, the growth and luminescence of cells containing LEE/EsaR-D91G were measured in the absence of both IPTG and without *esaI*. As shown in Figure 4a, very low background levels of luminescence were observed throughout the growth period, indicating that the LEE promoter remains repressed throughout the growth period. We next added 1 mM IPTG to the same LEE/EsaR-D91G cells without *esaI* and found that the addition of the exogenous inducer alone did not lead to any detectable increase in luminescence (Figure 4a). Similarly, background luminescence levels were observed from LEE/EsaR-D91G cells with EsaI (LEE/EsaR-D91G/EsaI) in the absence of IPTG (Figure 4a). However, an increase in luminescence was observed when IPTG was added to the LEE/EsaR-D91G/EsaI cells, where luminescence above background was observed between 4 and 6 h and continued to increase throughout the growth period (Figure 4a). LEE/EsaR-V220A/EsaI cells also showed a QS-dependent increase in luminescence in the presence of exogenously added IPTG (Figure 4a). Consistent with our initial characterization of the LEE promoter in the presence of exogenously added IPTG and 3OC6HSL, the maximum level of luminescence observed with EsaR-D91G was 2-fold higher than that with EsaR-V220A (Figure 4a). Luminescence from LEE/EsaR-D91G/EsaI and LEE/EsaR-V220A/EsaI increased above background during early- to midlog phase (Figure 4a and b). The timing and magnitude of the observed increases in luminescence indicate that the concentration of 3OC6HSL in the media was at least 1 μM by midlog phase. The growth observed from each strain containing LEE was similar, indicating that the observed changes in gene expression were not due to differences in cell growth (Figure 4b).

The behavior of the TTE promoter was characterized as described for LEE above, except 100 ng/mL of aTc was added instead of IPTG. In TTE/EsaR-D91G cells without EsaI, background levels of luminescence were detected both with and without aTc (Figure 4c). For TTE/EsaR-D91G/EsaI, background luminescence was observed in the absence of aTc (Figure 4c). Upon addition of 100 ng/mL aTc, increased luminescence from TTE/EsaR-D91G/EsaI was observed during cell growth, where luminescence above background was observed between 6 and 8 h and continued to increase until the cultures reached stationary phase around 12 h (Figure 4c and d). The timing and magnitude of luminescence observed when EsaR-D91G was replaced by EsaR-V220A were similar (Figure 4c). As with LEE, these data indicate that the concentration of 3OC6HSL in the media reached 1 μM by midlog phase. This is consistent with our initial characterization of TTE with the two EsaR variants. Cell growth was similar for all TTE strains (Figure 4d).

Both the LEE and TTE promoters exhibited the desired QS-dependent increases in gene expression in the presence of an

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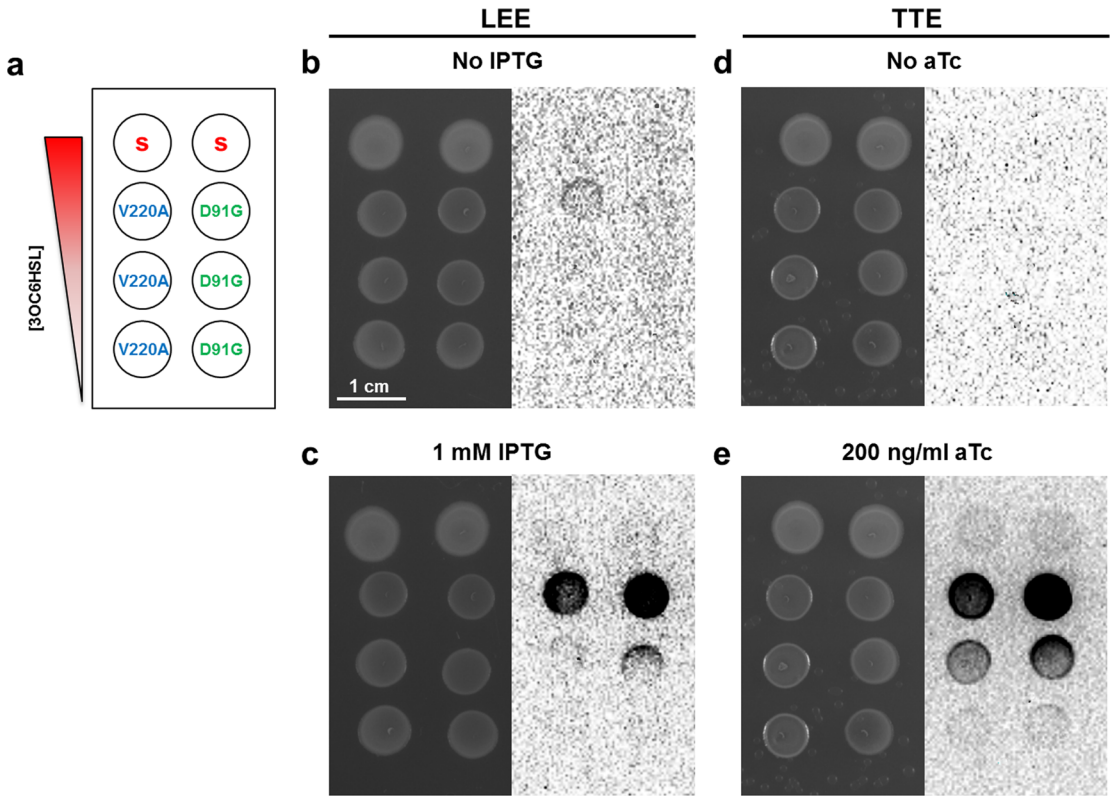

Figure 5. Cell−cell communication-dependent AND-gate behavior of LEE and TTE. (a) Schematic showing the placement of the sender cells (S), which produce 3OC6HSL, and the AND-gate containing cells, which contain either EsaR-V220A (V220A) or EsaR-D91G (D91G). Spots of each strain were generated by pipetting 1 μL of an overnight culture onto an LB agar plate. The distance between spots, center-to-center, is approximately 1 cm. The 3OC6HSL diffuses away from the sender cells and generates a concentration gradient. (b−d) Both pictures of the spots on the agar plates and luminescence images obtained using a ChemiDoc XRS+ System (Bio-Rad). The color and brightness of the luminescence images were inverted for clarity. Luminescence from LEE/EsaR-V220A and LEE/EsaR-D91G was determined (b) without IPTG and (c) in the presence of 1 mM IPTG. Luminescence from TTE/EsaR-V220A and TTE/EsaR-D91G was determined (d) without aTc and (e) in the presence of 200 ng/mL aTc.

exogenously added inducer. The maximum levels of luminescence observed from TTE were 3 to 6-fold higher than from LEE, consistent with our initial characterization of the promoters (Figure 4a and c). Although gene expression above background was observed between 4 and 6 h for LEE and between 6 and 8 h for TTE, these times correspond to midlog phase in both cases because the JM109 cells grew slightly faster than DH5α-PRO in our assays. The observation that gene expression is turned on during midlog phase indicates that these constructs may be useful alternatives to other autoinduction systems, where a QS system[38,39] or a diauxic shift in carbon source utilization[40] have been used to turn on gene expression as cell density increases in order to maximize protein expression without having to monitor cell growth and add an exogenous inducer at a specific time. In the case of the AND-gates described here, however, the second exogenously added inducer provides an extra level of control which could be used to allow cells to grow to high densities without protein expression or to modulate the level of QS-dependent expression.

AND Gate-Based Cell−Cell Communication. The AND gates may also be useful when gene expression is desired in the presence of both an exogenous inducer and a second cell strain or species. In this case, 3OC6HSL can be used as a cell−cell communication signal instead of as a QS signal. To this end, we examined whether strains containing LEE or TTE are able to respond to 3OC6HSL produced by a second cell strain and to the appropriate exogenously added inducer. The constitutive expression of *esaI* from a σ^{70}-dependent promoter on a plasmid in *E. coli* DH5α was used to generate a 3OC6HSL sender strain. Here, assays were conducted using cells grown on semisolid media in Petri dishes to investigate the response of the AND-gate strains to 3OC6HSL produced by the sender strain. Stationary phase culture of the sender cells (1 μL) was spotted onto LB agar plates (Figure 5a). The plates were incubated for 8 h at 37 °C and then 1 μL of the LEE or TTE cells was spotted 1, 2, and 3 cm away from the sender cells. The initial 8 h incubation time was included because it was observed to form a gradient of 3OC6HSL in the agar plates across the distance of the spots tested. Luminescence was observed following another 24 h growth at 37 °C.

As shown in Figure 5b, only background levels of fluorescence were observed from the spots containing LEE/EsaR-D91G or LEE/EsaR-V220A. However, when 1 mM IPTG was added to the agar plate, luminescence was observed from the spots closest to the sender cells containing either LEE/EsaR-V220A or -D91G (Figure 5c). Luminescence was also detected at the edge of the second LEE/EsaR-D91G spot closest to the sender cells, while luminescence was not observed from the second LEE/EsaR-V220A spot (Figure 5c). Luminescence was not observed at the third spot for either promoter. The observation that gene expression from LEE/EsaR-D91G is induced at lower concentrations of 3OC6HSL than LEE/EsaR-V220A is consistent with our initial characterization of the promoter and assays with endogenous 3OC6HSL production.

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Luminescence from TTE/EsaR-V220A and TTE/EsaR-D91G on agar plates with sender cells was observed in the absence of aTc (Figure 5d) and in the presence of 200 ng/mL aTc (Figure 5e). In the absence of aTc, fluorescence was not observed from any of the TTE-containing cells (Figure 5d). On the agar plate containing aTc, luminescence was detected from the entire TTE/EsaR-V220A and TTE/EsaR-D91G spots closest to the sender cells (Figure 5e). Luminescence was also observed from the second spot away from the senders for both TTE/EsaR-V220A and TTE/EsaR-D91G (Figure 5e). For TTE/EsaR-D91G, more luminescence was observed at the edge of the second spot closest to the sender cells, while luminescence from TTE/EsaR-V220A was lower and more uniform across the spot (Figure 5e). Although subtle, this is the first assay in which a difference in 3OC6HSL sensitivity was observed between EsaR-V220A and -D91G from TTE. As with LEE, luminescence was not observed from the third spot.

We have demonstrated that our QS-dependent AND-gate system enables cells to respond to an endogenous signal and an exogenously added signal. These promoters may serve as a template for designing new multi-input promoters that respond to 3OC6HSL and one or more additional molecular cues by combining the *esa* box and other transcriptional regulator binding sites. Our results with TTE suggest that a single *esa* box downstream of the −10 site is sufficient to enable repression and 3OC6HSL-dependent gene expression. New promoters that recognize multiple signals could be generated by adding transcription factor binding sites upstream of the −35 site and between the −35 and −10 site. Further, a combinatorial approach may be used to rapidly generate and characterize a library of synthetic promoters that can integrate specific inputs. The two-input promoters could also be used in combination with tandem promoters to further increase the number of inputs that can be recognized by a single cell. For example, two AND-gate promoters could be stacked together to build a tandem promoter that is able to integrate three or more inputs. Using tandem multi-input promoters to enable a 4-input logic gate response in a single cell would be complementary to the approach described by Moon et al.,[41] where they employed protein−protein interactions between the outputs of two tandem promoters to control gene expression from a third promoter, which served as the circuit output.

Although we have successfully shown that the EsaR/*esa* box pair can be used to enable 3OC6HSL-dependent gene expression at a non-native and in this case multi-input promoter, the use of this QS module in more complicated systems may be limited by the fact that placing the *esa* box upstream of the −35 site leads to activation of gene expression. This limitation may be addressed by using an EsaR variant that retains its ability to bind to the DNA but is not able to activate gene expression, regardless of the placement of the *esa* box. Several EsaR variants with decreased ability to activate gene expression have been described.[34] Subsequent studies may examine whether these variants are capable of repressing gene expression at a σ^{70}-dependent promoter, similar to our AND-gate promoters, where the *esa* box is placed upstream of the −35 site. Alternatively, the ability of EsaR to activate gene expression in the absence of 3OC6HSL could be used in combination with another activator or repressor to construct other types of logic gates, such as NAND, IMPLY, and NIMPLY gates, where turning off gene expression in the presence of 3OC6HSL is desired.

Here, we have shown that endogenous expression of an AHL synthase, EsaI, enables QS-dependent AND-gate behaviors from LEE and TTE, where gene expression increases as cell density increases only in the presence of an exogenous inducer. To enable dynamic changes in gene expression, which may be useful in a range of metabolic engineering applications, it may be useful to engineer cells to respond to both cell density and a second endogenous signal, such as the availability of a key metabolite, cofactor or toxic byproduct. For example, a metabolite can serve as an input to an AND-gate promoter that also contains an *esa* box so that some downstream metabolic reactions that yield products that inhibit cell growth, such as butanol production, occur only at high cell densities when nontoxic intermediates have accumulated in the cells. Finally, we envision that our AND gates will be useful for coordinating metabolic processes between cells in microbial consortia used for chemical production. In this case, a particular reaction, or even growth of one of the organisms in a community, could be controlled in response to both the population level of another organism by QS and the availability of a target metabolite.

METHODS

Plasmid Construction. The TTE and LEE promoters (Figure S1, Supporting Information) were based on the AND gate promoters described by Ramalingam et al.,[9] where TTE was generated by replacing the *lacO* in TTL with an *esa* box, and LEE was generated by replacing the two *tetO* in LTT with two *esa* boxes. The two promoters were made by DNA synthesis (Genscript) and provided in a pUC57 vector. LEE and TTE were cloned between the *Xho*I and *Bam*HI sites upstream of the luciferase operon, *luxCDABE,* in pCS26 to generate pLEE and pTTE. A constitutive, σ^{70}-dependent promoter was PCR-amplified from pCS-σ^{70}-*lux* (M. Surette, unpublished) using 5-σ^{70}-*Xba*I (tctagacgtctaagacaccactagtatcatg) and 3-σ^{70}-*Kpn*I (ggtacc tttctcctctttagcggccgcaactagaaattgcacgtagaatacgagccgg) and cloned into *Kpn*I and *Bam*HI-digested pAC-EsaR[36] to generate pAC-σ^{70}-EsaR. The same method was used to construct pAC-σ^{70}-EsaR-D91G and pAC-σ^{70}-EsaR-V220A from pAC-EsaR-D91G and pAC-EsaR-V220A. A codon-optimized *esaI* gene was PCR-amplified using 5-EsaI-*Bam*HI (ctgcaaggatccttcgcgagggccgcagtaactttt) and 3-EsaI-term-*Sal*I (ctgatc gtcgactcatctggatttgttcagaacgctc) from pAC-EsaR-EsaI[35] and cloned downstream of the *esaR* gene in *Bam*HI and *Xho*I-digested pAC-σ^{70}-EsaR to create pAC-σ^{70}-EsaR-EsaI. The same strategy was used to generate pAC-σ^{70}-EsaR-D91G-EsaI and pAC-σ^{70}-EsaR-V220A-EsaI. Table S1 in Supporting Information includes detailed information on all *E. coli* strains and plasmids.

Quantitative Characterization. The pLEE plasmid was transformed into *E. coli* JM109 with each of the following plasmids: pAC-σ^{70}-EsaR, and pAC-σ^{70}-EsaR-D91G, and pAC-σ^{70}-EsaR-V220A to generate LEE/EsaR, LEE/EsaR-D91G, and LEE/EsaR-V220A. The pTTE plasmid was similarly transformed into *E. coli* DH5α-PRO with the three EsaR expression vectors to generate TTE/EsaR, TTE/EsaR-D91G, and TTE/EsaR-V220A. Cells were cultured in the presence of chloramphenicol (50 μg/mL) and kanamycin (50 μg/mL) throughout the experiments detailed below. Gene expression was characterized as described previously,[36] in the presence of 0, 1, 10, 100, 1000, and 10 000 nM 3OC6HSL (Sigma). For LEE, gene expression in the presence of each concentration of 3OC6SHL was measured with 0, 0.01, 0.1, and 1 mM IPTG.

Similarly, TTE was characterized with each concentration of 3OC6HSL and 0, 10, 100, or 200 ng/mL aTc.

Shake Flask Assays. The pLEE plasmid was transformed into *E. coli* JM109 with each of the following plasmids: pAC-σ^{70}-EsaR-EsaI, and pAC-σ^{70}-EsaR-D91G-EsaI, and pAC-σ^{70}-EsaR-V220A-EsaI to generate LEE/EsaR-EsaI, LEE/EsaR-D91G-EsaI, and LEE/EsaR-V220A-EsaI. The pTTE plasmid was similarly transformed into *E. coli* DH5α-PRO with the three EsaR-EsaI expression vectors to generate TTE/EsaR-EsaI, TTE/EsaR-D91G-EsaI, and TTE/EsaR-V220A-EsaI. Overnight cultures were diluted 500-fold (v/v) into 50 mL Luria−Bertani (LB) medium containing appropriate antibiotics and the cultures were grown at 37 °C with shaking for 14 h. The pLEE-containing strains were assayed with 0 or 1 mM IPTG and the pTTE-containing strains were assayed with 0 or 100 ng/mL aTc. IPTG and aTC were added at the time of inoculation. Luminescence and optical density at 600 nM (OD_{600}) were measured using a Perkin-Elmer Envision plate reader every 2 h by transferring 200 μL to a black 96-well plate with a clear bottom.

Agar Plate Luminescence Assay. The 3OC6HSL-producing sender strain was generated by transforming pAC-σ^{70}-EsaR-EsaI and the promoterless vector, pCS26, into *E. coli* DH5α. An overnight culture of the sender strain (1 μL) was spotted onto an LB agar plates containing kanamycin and chloramphenicol. The plates were then incubated for 8 h at 37 °C. After 8 h of incubation, three 1 μL spots of LEE/EsaR-D91G, LEE/EsaR-V220A, TTE/EsaR-D91G, and TTE/EsaR-V220A were plated with a center-to-center distance of 1 cm between neighboring spots. For LEE strains, cells were assayed on agar without any inducer and with 1 mM IPTG, while TTE strains were assayed without inducer and with 200 ng/mL aTc. The agar plates were incubated at 37 °C for 24 h and screened using the ChemiDoc XRS+ System and Image Lab Software (Bio-Rad).

■ ASSOCIATED CONTENT

Supporting Information

Table S1, detailed information on all *E. coli* strains and plasmids; Figure S1, TTE and LEE promoters. 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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