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Engineering the *esaR* Promoter for Tunable Quorum Sensing-Dependent Gene Expression

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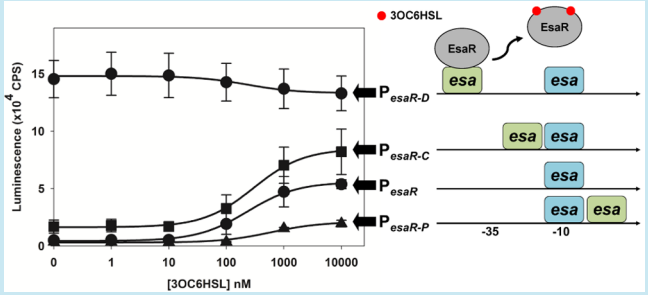
Supporting Information

ABSTRACT: Quorum sensing (QS) systems enable bacteria to coordinate their behavior as a function of local population density and are often used in synthetic systems that require cell−cell communication. We have engineered the *esaR* promoter, P_{esaR}, which is repressed by the QS regulator EsaR. EsaR-dependent gene expression from P_{esaR} is induced by 3-oxo-hexanoyl-homoserine lactone (3OC6HSL). Here, we report a set of modified P_{esaR} promoters that contain a second EsaR binding site. We observed changes in gene expression levels, regulatory range, 3OC6HSL sensitivity, and the regulatory role of EsaR that are dependent on the position of the second binding site. Combining the new promoters with endogenous 3OC6HSL production led to QS-dependent systems that exhibit a range of expression levels and timing. These promoters represent a new set of tools for modulating QS-dependent gene expression and may be used to tune the regulation of multiple genes in response to a single QS signal.



KEYWORDS: *quorum sensing, cell−cell communication, promoter engineering, gene expression, acyl-homoserine lactone*

Quorum sensing (QS), a natural form of cell−cell communication, regulates bacterial behaviors based on changes in their local cell population density.[1,2] As cell density increases, signal molecules accumulate and are sensed by regulators that modulate QS-controlled gene expression.[3] QS systems have been used in biotechnology and synthetic biology applications that require coordinated, community-level behaviors.[4,5] Further, the use of QS systems can negate the need to add exogenous inducers to turn on gene expression at a particular phase of cell growth. However, new tools and regulatory components are required to simplify the tuning of expression levels and timing needed for specific applications. Here, we focus on engineering the *esaR* promoter, P_{esaR}. which is repressed by the QS transcriptional regulator, EsaR.

Previous studies of inducible promoters have shown that the placement and spacing between transcription factor binding sites, generally known as operators, largely define how promoters are regulated.[6−11] For example, Meier et al. examined the roles of the two *tet* operators, O_1 and O_2, in controlling the divergently oriented TetR-regulated promoters, P_R and P_A.[12] They observed that mutating O_1, but not O_2, affected promoter activity and regulatory range, which is defined as the ratio of the induced to the uninduced promoter activity,[10] from P_R, while mutating either O_1 or O_2 affected promoter activity and regulatory range from P_A.[12] Oehler et al. observed that removing one or two of the three operators in the native *lac* promoter resulted in decreased regulatory range.[13,14] The location of the *lac* operator in both the native *lac* promoter and synthetic promoters has also been shown to alter regulatory range and expression levels.[6] Recently, synthetic σ^{54}-dependent promoters[15] and yeast *GAL1* promoters[9] containing multiple *tet* operators were observed to exhibit reduced minimum gene expression levels as the number of operators increased. Further, Cox et al., showed that the presence of two *lac* or *tet* operators in a set of synthetic combinatorial promoters led to a 10-fold increase in regulatory range.[10] These studies highlight the role that both the position and number of operators can play in modulating promoter activity. Overall, multiple copies of the same operator are predicted to increase the affinity of a transcription factor for a promoter, often decreasing minimum expression levels, while promoter activity can also be affected by the additional repressor binding sites through changes in RNA polymerase (RNAP) affinity[7,16] and DNA shape.[17−20] However, attempts at predicting the regulatory dynamics of multioperator promoters from the behaviors of single-operator promoters have indicated that the operators rarely function in a simple additive manner.[9,10]

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In this study, we examined the effect of adding a second EsaR binding site to P_{esaR}. EsaR binds to a specific 20-base pair (bp) DNA sequence of dyad symmetry known as an *esa* box. EsaR-dependent repression at P_{esaR} is caused by EsaR binding to an *esa* box that overlaps the −10 site.[21] Gene expression from P_{esaR} is induced in response to 3-oxo-hexanoyl-homoserine lactone (3OC6HSL), where 3OC6HSL binding causes EsaR to dissociate from the promoter.[21] EsaR can act as an activator at the *esaS* promoter, P_{esaS}.[22] EsaR-dependent activation at P_{esaS} is highest in the absence of 3OC6HSL and decreases following 3OC6HSL-dependent dissociation from the promoter. While the transcriptional start site and RNAP binding sites in the P_{esaS} promoter have not been identified, a 60 bp region downstream of the *esa* box appears to be sufficient for EsaR-dependent gene activation. Thus, whether EsaR behaves as an activator or a repressor likely depends on the position of the *esa* box in a promoter.

We previously used directed evolution to engineer EsaR variants with increased 3OC6HSL sensitivity.[23] However, the basal and fully induced levels of gene expression from P_{esaR} with each of the variants were similar. To expand our toolbox of repressor-based QS components, we generated and characterized promoters where a second *esa* box was added to P_{esaR}. We constructed three dual *esa* box promoters by adding a second *esa* box either upstream of the −35 site (distal), between the −35 and −10 sites (core), or directly upstream of the −35 site (proximal) in P_{esaR} to generate $P_{esaR\text{-}D}$, $P_{esaR\text{-}C}$, and $P_{esaR\text{-}P}$ respectively (Figure 1 and Figure S1, Supporting Information). In $P_{esaR\text{-}D}$, the *esa* box was added directly upstream of the −35 site. In $P_{esaR\text{-}C}$, the *esa* box was added between the −35 and −10 sites. In $P_{esaR\text{-}P}$, the *esa* box was placed eight nucleotide pairs downstream of the original *esa* box. In all cases, the spacing between the −35 and −10 sites, the transcriptional start site and the ribosome binding site were kept the same as in P_{esaR}. The dual *esa* box promoters were cloned upstream of the luciferase operon, *luxCDABE*, replacing the wild-type P_{esaR} in our previously characterized reporter plasmid, pCS-P_{esaR}*lux*,[23] which also contains an SC101 origin of replication (ori; 3−5 copies per cell) and a kanamycin resistance gene.

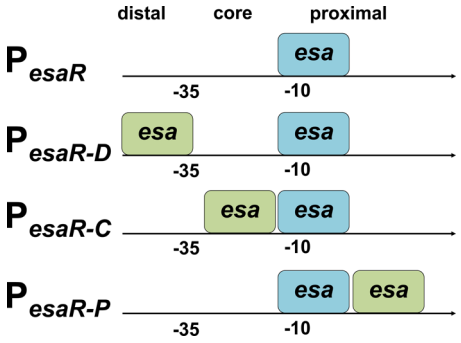

Figure 1. Illustration of the wild-type P_{esaR} promoter and dual *esa* box promoters. In addition to the original *esa* box (blue) in P_{esaR}, a second *esa* box (green) was added upstream of the −35 site, between the −35 and −10 sites, or downstream of the −10 site to construct $P_{esaR\text{-}D}$, $P_{esaR\text{-}C}$, and $P_{esaR\text{-}P}$, respectively. The promoters were cloned upstream of the *lux* operon in a plasmid with SC101 ori (3−5 copies per cell) or a pBR322 ori (30−50 copies per cell). The addition of a (+) to the promoter name indicates that is in the higher copy plasmid ($P_{esaR(+)}$, $P_{esaR\text{-}D(+)}$, $P_{esaR\text{-}C(+)}$, and $P_{esaR\text{-}P(+)}$).

To investigate how the additional *esa* box affects promoter behavior, we measured 3OC6HSL-dependent luminescence from each of the promoters in the absence of any regulator, or in the presence of wild-type EsaR or EsaR-D91G, which exhibits a 70-fold decrease in $K_{D(APP)}$ relative to EsaR.[23] Here, we define the apparent dissociation constant, $K_{D(APP)}$, as the concentration of 3OC6HSL required to achieve half-maximal luminescence. In the absence of EsaR, constitutive expression from P_{esaR} was observed regardless of 3OC6HSL concentration (Figure 2a). With wild-type P_{esaR} and EsaR, luminescence increased as a function of 3OC6HSL concentration, and luminescence was observed at lower concentrations of 3OC6HSL when EsaR was replaced by EsaR-D91G (Figure 2a). The minimum and maximum expression levels observed with EsaR and EsaR-D91G were similar, while the maximum expression level observed with 10 μM 3OC6HSL was significantly lower than that observed from the unregulated promoter.

In the absence of EsaR, $P_{esaR\text{-}P}$ showed a 10-fold decrease in luminescence compared to wild-type P_{esaR} (Figure 2b). Like the wild-type promoter, gene expression from $P_{esaR\text{-}P}$ was repressed by EsaR and EsaR-D91G, and similar concentrations of 3OC6HSL were required to induce gene expression (Table 1 and Figure 2b). Despite the decrease in promoter strength, the regulatory range was similar for P_{esaR} and $P_{esaR\text{-}P}$ (Table 1). In this case, the maximum luminescence levels observed with 10 μM 3OC6HSL were similar to the levels observed with the unregulated promoter.

Compared to P_{esaR}, a 2-fold increase in the promoter strength of $P_{esaR\text{-}C}$ was observed in the absence of EsaR (Figure 2c). Repression by EsaR and EsaR-D91G was observed at $P_{esaR\text{-}C}$. However, the maximum expression level observed with EsaR was approximately 2-fold less than with EsaR-D91G, which was about 50% less than from unregulated $P_{esaR\text{-}C}$. In addition, the $K_{D(APP)}$ value observed from $P_{esaR\text{-}C}$ increased 27-fold with EsaR-D91G relative to P_{esaR} (Table 1). The $K_{D(APP)}$ with wild-type EsaR was similar to that observed with wild-type P_{esaR}. However, this value may be underestimated because of the decreased range of expression levels observed.

Luminescence from $P_{esaR\text{-}D}$ in the absence of EsaR was similar to P_{esaR}. However, an increase in luminescence was observed from $P_{esaR\text{-}D}$ upon addition of EsaR or EsaR-D91G. Further, the addition of between 100 nM and 10 μM 3OC6HSL led to a decrease in luminescence (Figure 2d). These results indicate that the addition of a second *esa* box upstream of the −35 site in $P_{esaR\text{-}D}$ led to a promoter that is activated by EsaR, where the addition of 3OC6HSL decreases gene activation as the protein dissociates from the *esa* box. This was not entirely unexpected because EsaR has been shown to act as an activator at P_{esaS},[22] and the *lux* promoter, P_{luxI},[24] which is activated by the canonical QS activator LuxR in *Vibrio fisheri*.[25] However, EsaR is a much stronger activator at P_{esaS} relative to P_{luxI},[23] and this preference remains poorly understood. In $P_{esaR\text{-}D}$, it is also interesting to note that the second *esa* box added upstream of the −35 site that confers gene activation by EsaR appears to dominate any repression of gene expression by EsaR at the native *esa* box. Log−log plots of the gene expression data presented in Figure 2 are included as Figure S2 (Supporting Information). These plots enable easy comparison of the activities of P_{esaR} and the three dual *esa* box promoters as a function of 3OC6HSL concentration on same *y*-axis.

To examine how the expression and regulation of P_{esaR} and our dual *esa* box promoters are affected by increasing the

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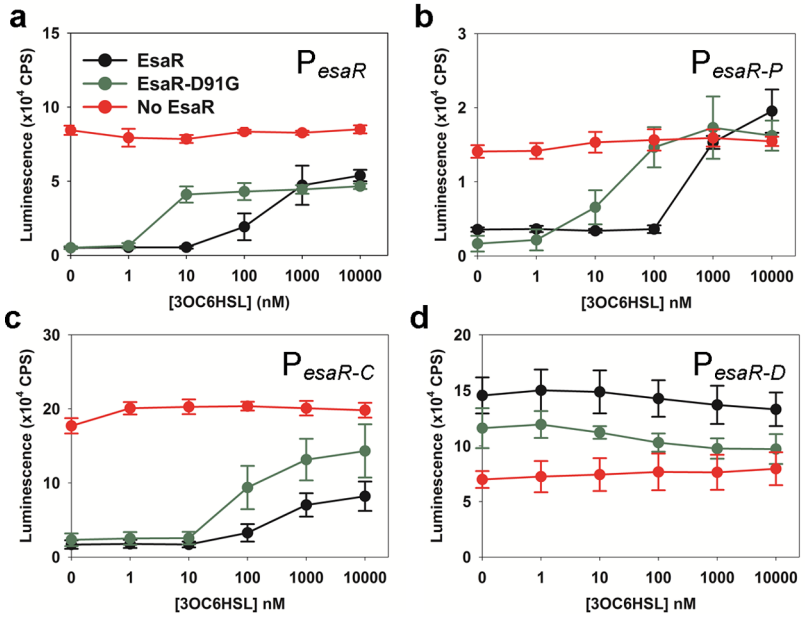

Figure 2. Gene expression from (a) P_{esaR}, (b) $P_{esaR\text{-}P}$, (c) $P_{esaR\text{-}C}$, and (d) $P_{esaR\text{-}D}$. Luminescence levels from each promoter were determined as a function of 3OC6HSL concentration without any regulator, with EsaR and with EsaR-D91G. Error bars show standard deviations from three independent biological replicates. Log–log plots of the same data with a consistent *y*-axis are included as Figure S2 (Supporting Information).

Table 1. 3OC6HSL Sensitivity, Uninduced and Induced Luminescence Levels, and Regulatory Range of Engineered Promoters with EsaR or EsaR-D91G

			luminescence (CPS)			
	EsaR type	$K_{D(APP)}$	no 3OC6HSL	10 μM 3OC6HSL	relative expression level[a]	regulatory range[b]
P_{esaR}	wild type	210 ± 110	5.0×10^3	5.4×10^4	1	10
	D91G	2.8 ± 0.6	5.0×10^3	5.0×10^4	1	10
$P_{esaR\text{-}P}$	wild type	680 ± 260	3.6×10^3	2.0×10^4	0.4	6
	D91G	20 ± 4	1.4×10^2	1.6×10^4	0.3	11
$P_{esaR\text{-}C}$	wild type	260 ± 20	1.7×10^4	8.2×10^4	1.5	5
	D91G	80 ± 20	2.3×10^4	1.4×10^5	3	6
$P_{esaR\text{-}C(+)}$	wild type	NA[c]	1.8×10^5	1.9×10^5	NA	NA
	D91G	12 ± 2	1.2×10^5	4.0×10^5	7	3

[a]Ratio of luminescence at 10 μM 3OC6HSL for a given promoter/repressor combination to P_{esaR} with wild-type EsaR at 10 μM 3OC6HSL. [b]The luminescence value at 10 μM divided by that without 3OC6HSL. [c]Not applicable.

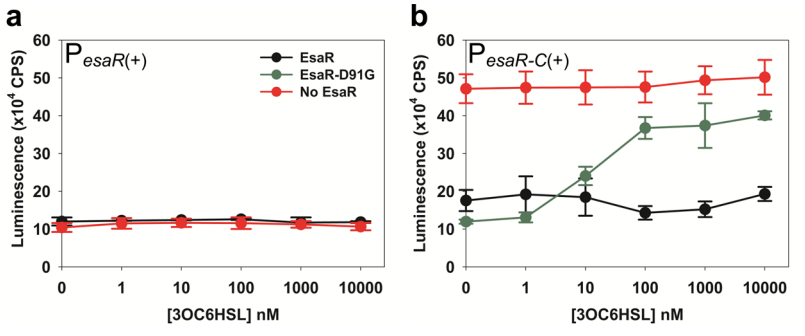

Figure 3. Gene expression from $P_{esaR(+)}$ and $P_{esaR\text{-}C(+)}$ on a medium copy number plasmid. Luminescence levels from each promoter were determined as a function of 3OC6HSL concentration without any regulator, with EsaR and with EsaR-D91G. Error bars show standard deviations from three independent biological replicates.

number of promoters per cell, we characterized them in a vector containing a pBR322 ori (30–50 copies per cell). Both the promoters and *lux* operon were cloned into pET17b and were characterized as described above for our low copy constructs. This was motivated by our finding that with wild-type P_{esaR} on a pET plasmid, or $P_{esaR(+)}$, constitutive luminescence was observed even with EsaR, and regardless of 3OC6HSL concentration (Figure 3a). We hypothesized that EsaR-dependent regulation was possible from our dual *esa* box promoters because of the potential increase in EsaR affinity due to the additional operator site.

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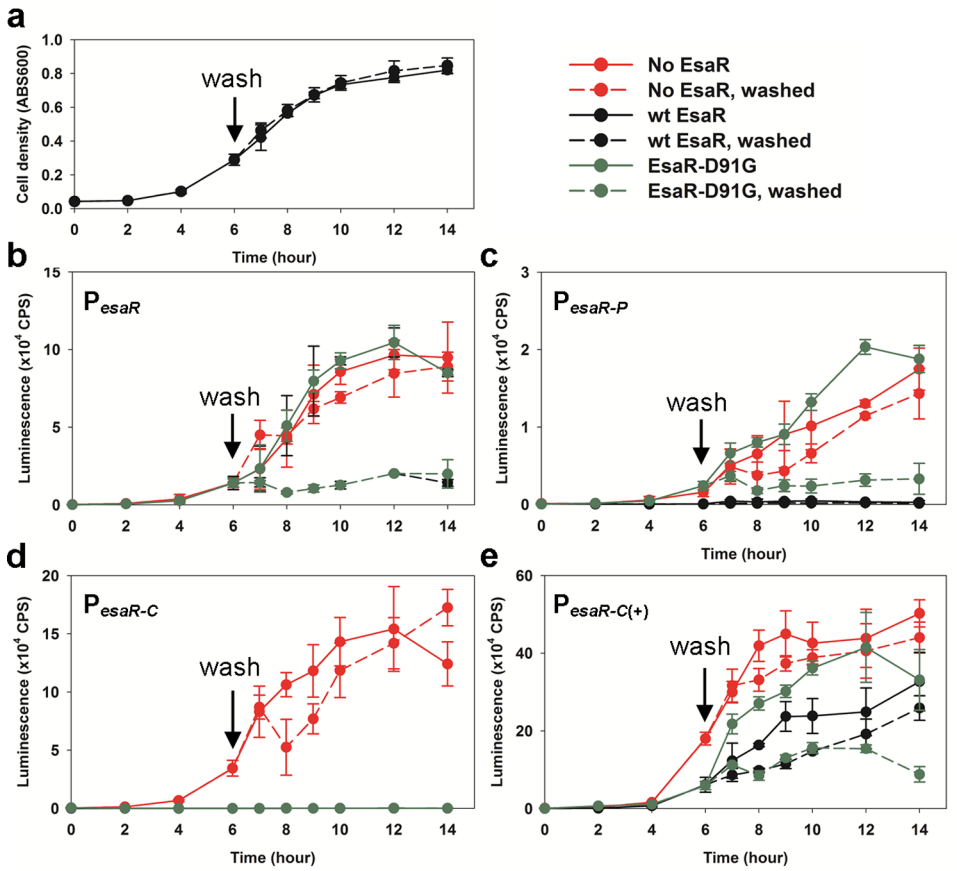

Figure 4. EsaR-dependent gene expression from the wild-type and engineered P_{esaR} promoters during batch growth with exogenously added 3OC6HSL. Cell growth and luminescence were measured during batch growth in a shake flask containing LB media and 1 μM 3OC6HSL. At the 6 h time point, half of each culture was washed and resuspended in fresh LB media without 3OC6HSL. Each promoter was assayed with wild-type EsaR or EsaR-D91G, and in the absence of any EsaR regulator (no EsaR). There were no significant differences between the growth curves obtained with all strains in the assay, regardless of washing. (a) A representative growth curve showing the average OD_{600} values from each strain containing wild-type EsaR. Growth of each individual strain is shown in Figure S5 (Supporting Information). Luminescence from (b) P_{esaR}, (c) $P_{esaR\text{-}P}$, (d) $P_{esaR\text{-}C}$, and (e) $P_{esaR\text{-}C(+)}$ from inoculation to stationary phase are shown. Error bars show standard deviations from three independent biological replicates.

Luminescence observed because of constitutive expression of the *lux* operon from $P_{esaR\text{-}C}$ on a pET vector, $P_{esaR\text{-}C(+)}$, in the absence of EsaR was 2-fold higher than that from $P_{esaR\text{-}C}$ and 5-fold higher than that from wild-type $P_{esaR(+)}$ (Figure 3a and b). While we observed a decrease in luminescence in the presence of EsaR, de-repression of $P_{esaR\text{-}C(+)}$ was not observed with 3OC6HSL concentrations as high as 10 μM (Figure 3b). However, we observed both repression and 3OC6HSL-dependent induction of gene expression with EsaR-D91G (Figure 3b). The 3OC6HSL sensitivity, $K_{D(APP)}$, of EsaR-D91G decreased from 80 to 12 nM with $P_{esaR\text{-}C(+)}$ relative to $P_{esaR\text{-}C}$ (Table 1). While the regulatory range observed from $P_{esaR\text{-}C(+)}$ was lower than from $P_{esaR\text{-}C}$, we have demonstrated that an additional binding site may be used to increase the affinity of EsaR for a promoter and enable inducible expression from a higher copy vector.

A 2-fold increase in promoter strength of $P_{esaR\text{-}P(+)}$ was observed relative to that of $P_{esaR\text{-}P}$ (Figure S3, Supporting Information). However, cells containing $P_{esaR\text{-}P(+)}$ grew very slowly, if at all, in the presence of wild-type EsaR or EsaR-D91G. No growth defects were observed from cells containing $P_{esaR\text{-}P(+)}$ alone. We had previously observed a similar growth defect from cells expressing a codon-optimized EsaR, which was generated with the goal of increasing EsaR production (data not shown). Together, these observations suggest that overexpression of EsaR may lead to toxicity or cause decreases in growth rate by an as yet undetermined mechanism. As with $P_{esaR\text{-}D}$, luminescence from $P_{esaR\text{-}D}$ on the pET plasmid, $P_{esaR\text{-}D(+)}$, showed high background luminescence in the absence of EsaR and was activated by EsaR or EsaR-D91G (Figure S4, Supporting Information).

While our final goal for this work was to examine how the engineered promoters behave when 3OC6HSL is produced endogenously and gene expression is induced in a QS-dependent manner, we first examined how gene expression from each promoter changes during batch culture growth when 1 μM 3OC6HSL is added exogenously at the time of inoculation. Because the luminescence output will increase both because of changes in gene expression and because of increased cell numbers, this set of data provides a baseline for the behavior of these promoters during batch growth. Optical density and luminescence from strains containing P_{esaR}, $P_{esaR\text{-}P}$, $P_{esaR\text{-}C}$, or $P_{esaR\text{-}C(+)}$ were measured as a function of time, from inoculation to stationary phase, in the absence of EsaR, with wild-type EsaR, or with EsaR-D91G. We did not include the weakly activated promoter, $P_{esaR\text{-}D}$, in these assays because of its high basal levels of gene expression.

Optical density measurements for all strains were very similar, indicating that cell growth is minimally affected by gene expression in these assays. To allow easy comparisons between

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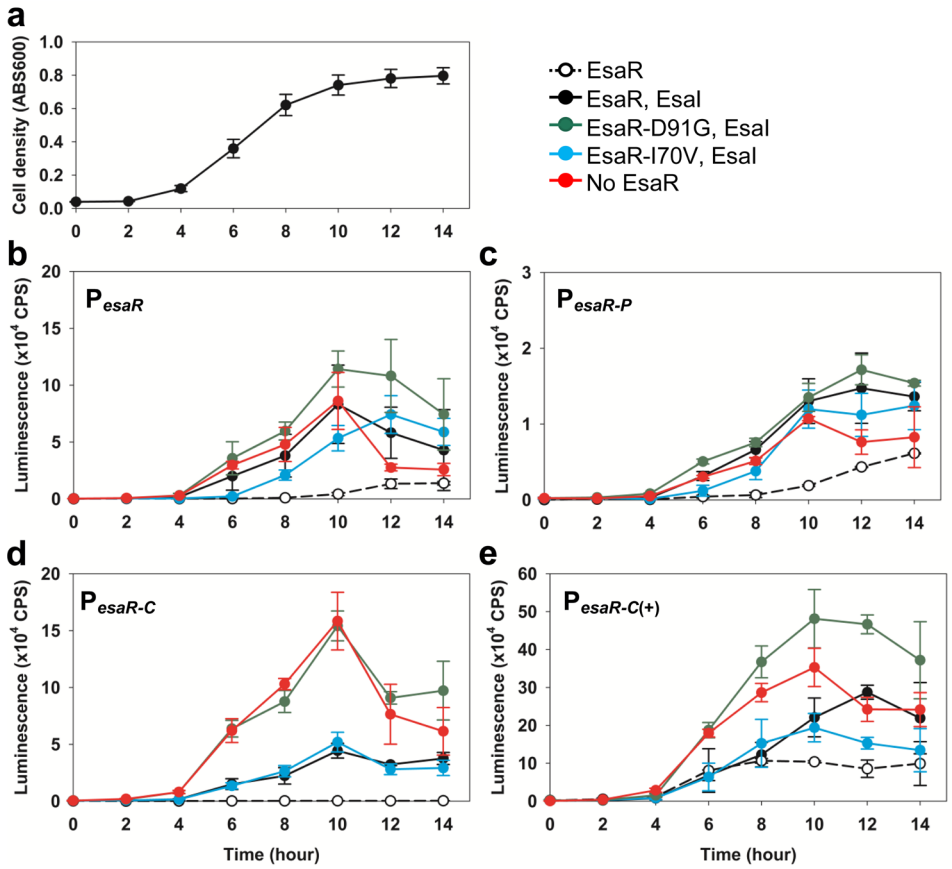

Figure 5. Endogenous 3OC6HSL production by the AHL synthase EsaI enables EsaR- and density-dependent gene expression from the wild-type and engineered P_{esaR} promoters. Cell growth and luminescence were measured during batch growth in a shake flask. Each promoter was assayed with wild-type EsaR, with EsaI and wild-type EsaR, EsaR-D91G, or EsaR-I70V, and in the absence of any regulator (No EsaR). There were no significant differences between the growth curves obtained with all strains in the assay. (a) A representative growth curve showing the average OD_{600} values from each strain containing wild-type EsaR and EsaI is shown. Growth of each individual strain is shown in Figure S6 (Supporting Information). Luminescence from (b) P_{esaR}, (c) $P_{esaR\text{-}P}$, (d) $P_{esaR\text{-}C}$, and (e) $P_{esaR\text{-}C(+)}$ from inoculation to stationary phase are shown. Error bars indicate standard deviations from three independent biological replicates.

growth and gene expression data, a representative growth curve, which is the average of the OD_{600} values from each of the four different promoter-containing strains with wild-type EsaR, is shown in Figure 4a. All individual growth curves are shown in Figure S5 (Supporting Information). In the absence of EsaR, each of these promoters is expected to behave as a constitutive promoter. Therefore, the output from these strains should provide an upper bound on gene expression, where luminescence increases primarily because the number of cells in the culture increases. For each of the promoters, the maximum level of gene expression observed during batch culture growth (Figure 4b–e) was consistent with the results from our initial characterization of the promoters (Figures 2a–c and 3b). As expected, luminescence increased during culture growth and leveled off as the cells approached stationary phase.

Similar levels of gene expression were observed from P_{esaR} in the presence of 3OC6HSL and either EsaR or EsaR-D91G relative to the unregulated promoter (Figure 4b). To verify that 3OC6HSL is required for gene expression in these strains, half of each cell culture was washed once and resuspended in fresh medium lacking 3OC6HSL. As shown by the dashed lines in Figure 4b, the removal of 3OC6HSL did not significantly effect gene expression from P_{esaR} in the absence of EsaR. However, luminescence from cells containing EsaR or EsaR-D91G rapidly declined following the removal of 3OC6HSL. This shows that 3OC6HSL is required for gene expression from P_{esaR} in the presence of EsaR or an EsaR variant, and that 3OC6HSL is required for sustained gene expression during the cell culture.

As expected, gene expression from $P_{esaR\text{-}P}$ was approximately 5-fold lower than from the wild-type promoter, and washing of the cells to remove 3OC6HSL led to a decrease in luminescence from cells containing EsaR-D91G but not in cells without any EsaR protein (Figure 4c). However, no increase in luminescence was observed from $P_{esaR\text{-}P}$ with wild-type EsaR (Figure 4c). This was unexpected because we had previously observed luminescence from $P_{esaR\text{-}P}$ with EsaR in the presence of 1 μM 3OC6HSL (Figure 2b). The major difference between these two assays is the timing of 3OC6HSL addition, where the cells in our initial assays were exposed to 3OC6HSL during midlog phase instead of at the time of inoculation. We observed a similar effect at $P_{esaR\text{-}C}$. In this case, increased luminescence was only observed in the absence of EsaR and no increase in luminescence was observed with EsaR or EsaR-D91G in the presence of 1 μM 3OC6HSL (Figure 4d). These results suggest that the timing of 3OC6HSL addition may affect sensitivity to the inducer. Degradation of 3OC6HSL is not expected to be significant during culture growth. However, the promoter/repressor combinations with higher $K_{D(APP)}$ values remain unaffected by the addition of 3OC6HSL, indicating that degradation of 3OC6HSL cannot be ruled out. Cells containing

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$P_{esaR\text{-}C}$ and EsaR or EsaR-D91G cultured with 10 μM 3OC6HSL did not grow (data not shown). Together with the observation that $P_{esaR\text{-}P(+)}$ grows very poorly in the presence of EsaR, these results suggest that EsaR, or another component of this system, is capable of causing toxicity under some conditions via an as yet unknown mechanism.

As expected, an increase in luminescence was observed from $P_{esaR\text{-}C(+)}$ in the absence of EsaR during culture growth (Figure 4e), where the maximum expression level was four to 5-fold higher than from wild-type P_{esaR}. Unlike $P_{esaR\text{-}C}$, increased luminescence was also observed from the strains containing $P_{esaR\text{-}C(+)}$ with EsaR or EsaR-D91G (Figure 4e). Similar to the results from the initial assays of $P_{esaR\text{-}C(+)}$ with EsaR (Figure 3b), the basal level of expression was high and washing the cells to remove 3OC6HSL did not substantially affect the level of gene expression (Figure 4e). However, $P_{esaR\text{-}C(+)}$ with EsaR-D91G showed higher levels of expression than with wild-type EsaR and a significant decrease in gene expression following washing to remove the 3OC6HSL (Figure 4e). This result is also consistent with our initial characterization of $P_{esaR\text{-}C(+)}$, indicating that the combination of $P_{esaR\text{-}C(+)}$ with EsaR-D91G may function well as part of a QS system where 3OC6HSL is produced endogenously.

As described above, QS systems enable bacteria to regulate gene expression as a function of cell density in response to QS signal molecules. To investigate QS-mediated gene expression using the new promoters, we cloned wild-type *esaI*, which encodes a 3OC6HSL synthase, downstream of *esaR* to enable constitutive production of 3OC6HSL. We examined gene expression from P_{esaR}, $P_{esaR\text{-}P}$, $P_{esaR\text{-}C}$, and $P_{esaR\text{-}C(+)}$ in the absence of EsaR (no EsaR), with EsaR alone, and with EsaI and either wild-type EsaR or an EsaR variant. Similar to the experiments described above, optical density and luminescence of batch cultures grown in shake flasks were measured every 2 h from inoculation to stationary phase. However, for these assays 3OC6HSL was not added exogenously. There was no significant difference between the optical density measurements of any of the promoter-containing strains, regardless of the presence of EsaR or EsaI, indicating that the observed changes in gene expression, which are described below, cannot be attributed to differences in cell growth (Figure 5a and Figure S6, Supporting Information). As described above, gene expression from cells containing the wild-type and engineered P_{esaR} promoters in the absence of any EsaR protein is expected to be constitutive and increases in luminescence during cell growth primarily because of the increasing numbers of cells. The maximum level of gene expression observed from the unregulated promoters during batch culture growth (Figure 5b–e) was consistent with the results from the batch culture experiments where 3OC6HSL was added exogenously (Figure 4b–e).

While the luminescence observed from cells containing P_{esaR} without EsaR increased during cell growth, the addition of EsaR decreased gene expression to background levels indicating near complete repression of gene expression from the promoter (Figure 5b). In the presence of EsaR, luminescence levels slightly above background were only observed between 12 and 14 h (Figure 5b). Luminescence from the strain expressing both EsaR and EsaI was similar to the unregulated strain throughout the growth period (Figure 5b), where gene expression above background was observed between 4 and 6 h and the maximum level of luminescence was consistent with our previous assays. When wild-type EsaR was replaced by EsaR-D91G, a similar pattern of increased QS-dependent gene expression was observed. However, the maximum luminescence level observed was slightly higher than unregulated P_{esaR} (Figure 5b). In addition to EsaR-D91G, another EsaR variant, EsaR-I70V,[23] which exhibits 14-fold lower 3OC6HSL sensitivity than wild-type EsaR, was also used to examine the effect of reduced 3OC6HSL sensitivity on QS-mediated gene expression from the promoters. With EsaR-I70V, the induction time and the time at which peak luminescence was observed were delayed by about 2 h relative to wild-type EsaR (Figure 5b). However, the maximum expression level was similar to wild type (Figure 5b).

An increase in luminescence above background was observed from the strain containing $P_{esaR\text{-}P}$ and wild-type EsaR after 10 h of growth (Figure 5c), indicating that gene expression from this promoter is more leaky than from the wild-type promoter. QS-dependent gene expression was also observed from $P_{esaR\text{-}P}$ with EsaI and EsaR or EsaR-D91G, where the level and timing of luminescence were similar to those observed with the unregulated promoter (Figure 5c). As with the wild-type promoter, EsaR-I70V delayed the induction time by approximately 2 h. While the overall pattern of expression was similar to what we observed with P_{esaR}, the maximum expression levels observed from $P_{esaR\text{-}P}$ were approximately 10-fold lower (Figure 5c).

Unlike P_{esaR} and $P_{esaR\text{-}P}$, no luminescence was detected from the strain containing $P_{esaR\text{-}C}$ with wild-type EsaR alone. This suggests that the additional *esa* box in $P_{esaR\text{-}C}$ may increase EsaR-dependent repression, minimizing leaky expression and improving regulatory control (Figure 5d). Although gene expression was not observed from $P_{esaR\text{-}C}$ with either EsaR or EsaR-D91G when 1 μM 3OC6HSL was added exogenously at the time of inoculation (Figure 4d), endogenous production of 3OC6HSL by EsaI enabled QS-dependent gene expression from $P_{esaR\text{-}C}$ with either EsaR or EsaR-D91G (Figure 5d). Interestingly, two distinct density-dependent expression patterns were observed. The maximum luminescence levels observed from strains containing the unregulated promoter or expressing both EsaR-D91G and EsaI were more than 3-fold higher than those observed with wild-type EsaR or EsaR-I70V (Figure 5d). The difference in maximum expression level between wild-type EsaR and EsaR-D91G is similar to what was observed in the bioassay where exogenous 3OC6HSL was titrated (Figure 2c). In this case, the timing of induction was similar for all EsaR variants.

The strain with $P_{esaR\text{-}C(+)}$ and wild-type EsaR showed basal luminescence levels more than 10-fold higher than from P_{esaR}, $P_{esaR\text{-}P}$, and $P_{esaR\text{-}C}$ (Figure 5e). As expected, QS-dependent gene expression was observed from this promoter with EsaR-D91G and EsaI, where the maximum expression level was approximately 5-fold higher than from $P_{esaR\text{-}C}$. QS-dependent gene expression was also observed with wild-type EsaR and EsaI (Figure 5e). This was unexpected because no induction was observed from $P_{esaR\text{-}C(+)}$ in the presence of EsaR and up to 10 μM exogenously added 3OC6HSL (Figure 3b). This increase in expression could be due to EsaI expression leading to 3OC6HSL concentrations that are higher than 10 μM or due to the better coordination of growth and expression. At $P_{esaR\text{-}C(+)}$, the sensitivity of the EsaR variants correlated with the maximum level of expression observed, where the strain containing EsaR-D91G had the highest level of gene expression and EsaR-I70V the lowest (Figure 5e). Like $P_{esaR\text{-}C}$, the timing of induction was similar for all strains, regardless of the EsaR variant.

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As expected, different levels of QS-dependent gene expression were obtained from P_{esaR} and each of the dual *esa* box promoters in the presence of EsaR. At both the wild-type promoter and $P_{esaR\text{-}P}$, the identity of the EsaR variant had a relatively small effect on maximum expression levels. However, the repressor with the lowest 3OC6HSL sensitivity, EsaR-I70V, led to a delay in induction time at these two promoters. At $P_{esaR\text{-}C}$, in both the low and medium copy vectors, the identity of the EsaR variant altered maximum expression levels, while the timing of expression was unaffected.

For each of the promoters, endogenous 3OC6HSL production enabled levels of EsaR or EsaR-D91G-dependent gene expression comparable to the unregulated promoters. Further, the maximum levels of gene expression observed at P_{esaR}, $P_{esaR\text{-}P}$ and $P_{esaR\text{-}C(+)}$ were higher when gene expression was controlled by EsaR-D91G than from the unregulated promoter. This result indicates that some subtle differences in the timing of expression may be affecting metabolic load or growth rates at early time points, and indicates that QS-dependent gene expression may be used to obtain expression levels greater than unregulated systems.

Overall, we have engineered and characterized a set of EsaR-modulated promoters that enable tunable QS-dependent gene expression by adding a second EsaR binding site to the wild-type promoter. Like previous efforts to modulate promoter activity by adding additional operators,[11] our findings indicate that the position of the second transcription factor-binding site can affect promoter behaviors, including expression levels and signal sensitivity. However, we also observed that the position of the *esa* box affects whether the promoter is activated or repressed by EsaR.

Although additional operator sites have been shown previously to increase regulatory range,[9,10] this was not the case with the dual *esa* box promoters in the low copy plasmids. This suggests that the differences in expression observed from $P_{esaR\text{-}C}$ and $P_{esaR\text{-}P}$, relative to the wild-type promoter, may be due to changes in DNA sequence or structure that affect transcription rates and not directly due to increasing the affinity of EsaR for the promoter. However, our observation that adding an additional binding site to the wild-type promoter led to the generation of an EsaR-dependent, 3OC6HSL inducible system that functions in a higher copy, pBR322 ori, containing plasmid does indicate that the additional *esa* box increases the affinity of EsaR for $P_{esaR\text{-}C}$. We note that in addition to the expected increase in expression levels obtained by increasing the plasmid copy number, the generation of promoters that function on plasmids with different replication origins increases the flexibility of our system.

With endogenous production of 3OC6HSL, combining the new promoters with different EsaR proteins enabled a wide range of QS-dependent behaviors that could not be achieved by targeting only the regulatory protein. Although subtle differences in timing were observed, additional approaches, such as modulating EsaI expression or sigma factor binding, may be used to further tune when density-dependent induction of gene expression occurs. A recent study by Sharon et al., where they efficiently identified and characterized the effects of several regulatory elements in a promoter library, has provided new insights into how multiple factors are combinatorially responsible for controlling gene expression and regulation.[11] We envision our system may be expanded or optimized using insights gained from such efforts, likely in combination with directed evolution and high-throughput screening approaches, and targeting promoter components in addition to operators. Overall, the new promoters and QS-dependent systems described here may be useful for metabolic engineering applications where the different promoters and a single QS regulator could be used to optimize both the timing and expression of multiple genes in a complex pathway.

METHODS

Plasmid Construction. The $P_{esaR\text{-}D,\ C,\ P}$ promoters (Figure S1, Supporting Information) were generated by DNA synthesis (GenScript) and were provided in a pUC57 vector. The new promoters were PCR-amplified using 5-pUC57-f (gtaaaacgacggccagtg) and 3-pUC57-r (ggaaacagctatgaccatg) and cloned into *Xho*I and *Bam*HI digested pCS-P_{esaR}*lux*[23] using standard molecular biology methods to generate pCS-$P_{esaR\text{-}D}$*lux*, pCS-$P_{esaR\text{-}C}$*lux* and pCS-$P_{esaR\text{-}P}$*lux*. The promoter, reporter and terminators were cloned between the *Xho*I and *Bgl*II sites in pET17b (Novagen) to generate pET-P_{esaR}*lux*, pET-$P_{esaR\text{-}D}$*lux*, pET-$P_{esaR\text{-}C}$*lux*, and pET-$P_{esaR\text{-}P}$*lux*. A codon-optimized *esaI* was generated by DNA synthesis (GenScript) and cloned downstream of *esaR* in *Bam*HI and *Sal*II digested pAC-EsaR[23] to generate pAC-EsaR-EsaI. The same procedure was used to construct pAC-EsaR-I70V-EsaI and pAC-EsaR-D91G-EsaI.

Quantitative Characterization. Each pCS- and pET-$P_{esaR\text{-}D,C,P}$-*lux* plasmid was transformed into *E. coli* DH5α competent cells with each of the following plasmids: pACYC184, pAC-EsaR, and pAC-EsaR-D91G.[23] Gene expression was characterized using established protocols[23] in the presence of 0, 1, 10, 100, 1000, 10 000 nM 3-oxo-hexanoyl-DL-homoserine lactone (3OC6HSL, Sigma). Chloramphenicol (50 μg/mL) together with kanamycin (50 μg/mL) or ampicillin (100 μg/mL) were added for strains containing pAC and pCS or pET plasmids.

Shake Flask Assays. The pCS-P_{esaR}, $P_{esaR\text{-}C}$, $P_{esaR\text{-}P}$*lux* and pET-$P_{esaR\text{-}C}$*lux* plasmids were each transformed into DH5α competent cells with each of the following plasmids: pACYC184, pAC-EsaR, and pAC-EsaR-D91G. Density-dependent behaviors were assessed using established protocols.[26] Briefly, cells were grown overnight in Luria–Bertani (LB) medium containing appropriate antibiotics at 37 °C with shaking. Overnight cultures were diluted 500-fold (v/v) into 80 mL of LB medium containing appropriate antibiotics and 1 μM 3OC6HSL, and grown at 37 °C with shaking. After 6 h, 40 mL of culture from each flask was centrifuged (4000*g*, 4 °C) and resuspended in 40 mL of fresh LB medium. This step was repeated once, and the resuspended cultures were transferred to sterile flasks and cultured at 37 °C with shaking alongside the flasks containing the unwashed cells. Measurements of cell density and luminescence were taken every 1–2 h from inoculation to stationary phase by transferring 200 μL of inoculum from each flask to a black 96-well plate with a clear bottom and measured using a Perkin-Elmer Envision plate reader. Similar culture and growth conditions were used for shake flask assays for cells endogenously producing 3OC6HSL. However, cells were not washed and no 3OC6HSL was added. To generate the strains for the QS assays, the pCS-P_{esaR}, $P_{esaR\text{-}C}$, $P_{esaR\text{-}P}$*lux* and pET-$P_{esaR\text{-}C}$*lux* plasmids were each transformed into DH5α competent cells with each of the following plasmids: pACYC184, pAC-EsaR-I70V-EsaI, and pAC-EsaR-D91G-EsaI.

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■ ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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