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Evolved Quorum Sensing Regulator, LsrR, for Altered Switching Functions

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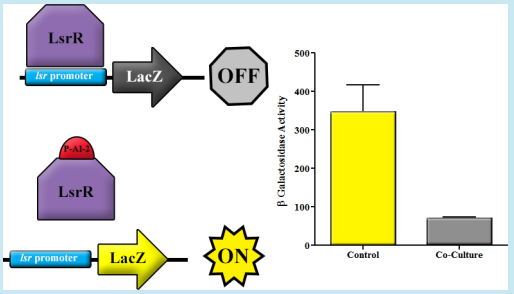
Supporting Information

ABSTRACT: In order to carry out innovative complex, multistep synthetic biology functions, members of a cell population often must communicate with one another to coordinate processes in a programmed manner. It therefore follows that native microbial communication systems are a conspicuous target for developing engineered populations and networks. Quorum sensing (QS) is a highly conserved mechanism of bacterial cell−cell communication and QS-based synthetic signal transduction pathways represent a new generation of biotechnology toolbox members. Specifically, the *E. coli* QS master regulator, LsrR, is uniquely positioned to actuate gene expression in response to a QS signal. In order to expand the use of LsrR in synthetic biology, two novel LsrR switches were generated through directed evolution: an "enhanced" repression and derepression eLsrR and a reversed repression/derepression function "activator" aLsrR. Protein modeling and docking studies are presented to gain insight into the QS signal binding to these two evolved proteins and their newly acquired functionality. We demonstrated the use of the aLsrR switch using a coculture system in which a QS signal, produced by one bacterial strain, is used to inhibit gene expression via aLsrR in a different strain. These first ever AI-2 controlled synthetic switches allow gene expression from the *lsr* promoter to be tuned simultaneously in two distinct cell populations. This work expands the tools available to create engineered microbial populations capable of carrying out complex functions necessary for the development of advanced synthetic products.



KEYWORDS: *quorum sensing, cell-to-cell communication, AI-2, directed evolution, switch, biosensor, LsrR*

Bacteria possess a variety of native regulatory circuits for sensing and responding to extracellular signals. These circuits are exquisitely tailored to specific signal molecules that initiate precise transduction cascades and cellular responses. The ability to engineer and exploit these circuits in designer organisms is fundamental to synthetic biology and its use in the development of biosensors and therapeutics, and the production of biofuels, pharmaceuticals, and novel biomaterials.[1] At the foundation of synthetic biology is a diverse, modular genetic toolbox that gives us the ability to harness and manipulate organisms to perform novel tasks and the expansion, and tunability of these tools is critical for advancement in this field. The development of new genetic tools is made challenging because understanding of these complex biological processes is limited and rational design approaches are often unsuccessful. Directed evolution is an alternative to rational design and provides an efficient strategy for developing new genetic modules or fine-tuning existing ones without an *a priori* knowledge of detailed functions, structural, or mechanistic information.[2,3]

In recent years, there has been increasing interest within the synthetic biology community to utilize engineered microbial consortia to perform complex functions that are not possible in individual strain populations. To accomplish this, members of a mixed microbial population must be able to communicate with one another in order to facilitate the division of labor necessary for multistep functions.[4] Quorum sensing (QS), a form of bacterial cell to cell communication, represents an ideal synthetic biology target. In addition to controlling population coordinated behaviors, the QS genetic circuitry is simple and intrinsically modular. In synthetic biology, QS circuits have emerged as promising tools for the design and synthesis of complex products.[5,6]

QS generally refers to the secretion and perception of autoinducer (AI) signal molecules that coordinate bacterial phenotypes, including virulence factor expression,[7−10] biofilm formation,[11] and biofouling.[12] There are a variety of recognized AI molecules, including QS molecule autoinducer 2 (AI-2).[13] Currently, over 70 bacterial species have been found to produce

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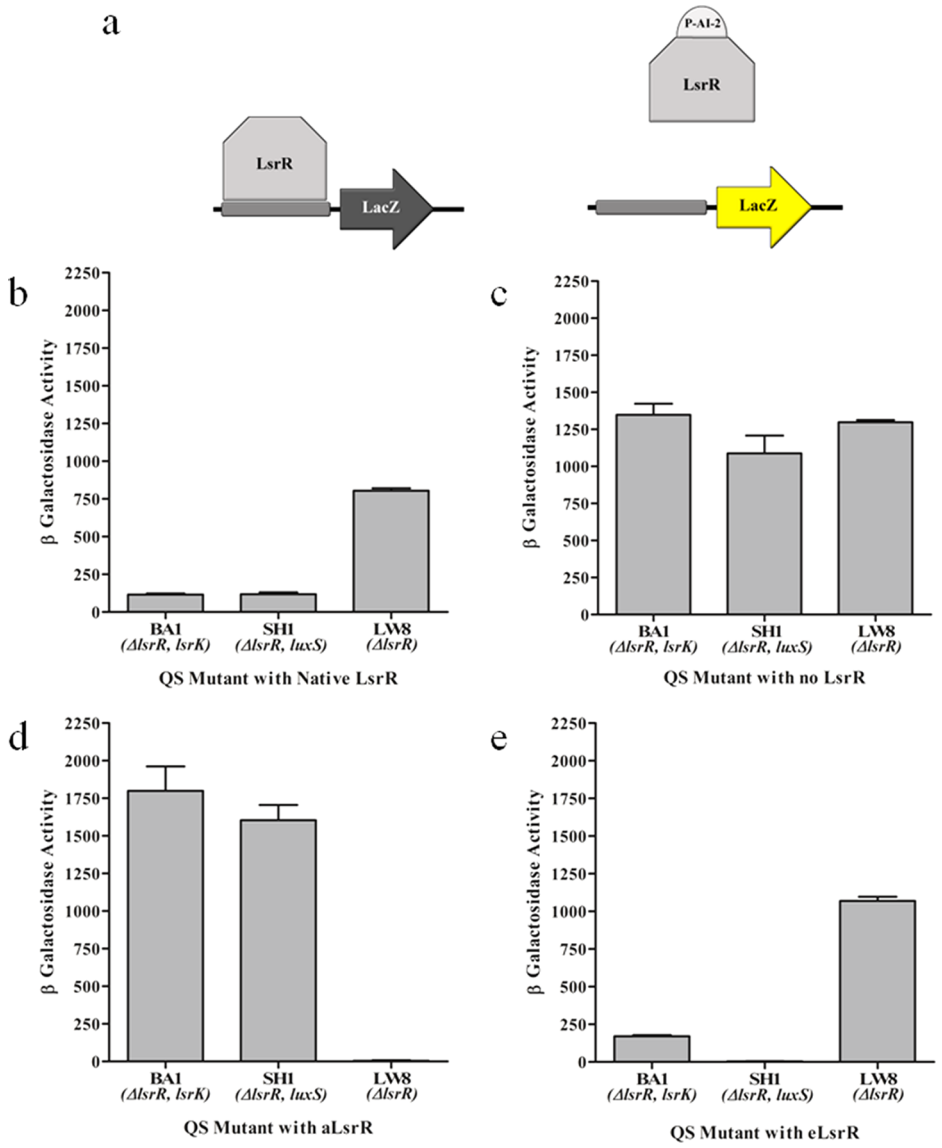

Figure 1. Evolution of novel LsrR transcriptional regulators. (a) Schematic of LsrR regulation of native gene expression (i.e., *lacZ*) from the *lsr* promoter. *β*-galactosidase production in various QS mutants containing plasmid-encoded (b) native LsrR, (c) no LsrR, (d) aLsrR, and (e) eLsrR.

this molecule,[13,14] and there are several others that do not themselves produce AI-2 but have been shown to respond to it.[9,15,16] Importantly, genes encoding the AI-2 transporter system are widespread among prokaryotes, indicating that many species are capable of AI-2 perception.[17] AI-2 is not a single discrete compound, but a family of 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran or furanosyl borate diester isomers derived from the intermediate molecule, DPD. AI-2 is biosynthesized via the activated methyl cycle and has been reviewed by Roy et al.[18] Briefly (as shown in Supporting Information (SI) Figure 1), the AI-2 synthase gene, *luxS*, converts S-ribosylhomocysteine (SRH) to 4,5-dihydroxy-2,3-pentanedione (DPD), which then cyclizes in solution to create the various AI-2 isoforms.[19,20] AI-2 is then thought to be transported outside the cell by the membrane spanning protein, TqsA (YdgG).[21] As the bacterial population density increases, the extracellular concentration of AI-2 also increases until a threshold is reached. At this time, AI-2 is transported back into the cell via the Lsr transporter and phosphorylated intracellularly by a kinase, LsrK. It is this phosphorylated form of AI-2 that binds to the repressor, LsrR, causing it to become destabilized and derepress the *lsr* operon expression.[22] In the absence of phosphorylated AI-2, LsrR binds the *lsr* promoter to prevent expression of the operon. The derepression of the *lsr* promoter initiates a positive feedback loop to facilitate the uptake of more AI-2.[23,24] LsrG is up-regulated as part of the *lsr* operon and functions to reduce intracellular phosphorylated AI-2 levels through degradation.[25] This ultimately leads to *lsr* operon repression, by reducing intracellular AI-2 levels and allowing LsrR to bind the *lsr* and *lsrRK* promoters.

Despite the pivotal role LsrR plays in the *E. coli* QS circuit, relatively little is known about this transcription regulator as compared to other systems (e.g., SorC[26]). Li et al.[27] showed that AI-2 controlled QS in *E. coli* through LsrR, presumably through the *lsr* promoter. Xue and colleagues[28] later empirically demonstrated that LsrR interacts with two 30 bp binding motifs in the *lsr* promoter region and phospho-AI-2 relieves this binding to activate transcription. The tertiary structure of LsrR was predicted by Roy et al.[29] using ESyPred3D and proposed it contained two domains, an HTH domain that was likely used

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for binding to DNA and a second domain, which was used for binding to phospho-AI-2. Preliminary crystallographic analysis also indicated that LsrR was a dimer consisting of two protein molecules per asymmetric unit.[30] The apo-LsrR crystal structure has been recently solved by Wu et al.,[22] showing (in agreement with the predicted structure[29]) it is composed of two domains: a N-terminal DNA-binding domain and a C-terminal ligand binding domain. Two LsrR molecules dimerize to form an asymmetric unit. In the absence of phospho-AI-2, two LsrR dimer units dimerize to form a tetramer (or dimer of dimers) and bind the *lsr* promoter. Wu et al. also showed that addition of phospho-AI-2 to the LsrR tetramer induces the dissociation of this protein into dimers. In addition to the *lsr* promoter, it is likely that LsrR has multiple DNA targets. Microarray analysis has shown that LsrR affects the expression of 67 genes, and an additional 79 genes in conjunction with LsrK.[27] Additionally, *S.* Typhimurium LsrR has been shown to be involved in the regulation of *Salmonella* pathogenicity island-1 genes to mediate virulence.[31] To date, only the *lsr* and *lsrR* promoter binding sites have been putatively identified in *E. coli*; these remain speculative due to the difficulties in carrying out comprehensive biophysical measurements with LsrR *in vitro*.

The exploitation of QS in synthetic biology and its inclusion in the biotechnology 'toolbox' has been recently reviewed by Tsao et al.[5] The *E. coli* QS circuit is a relatively tightly regulated system for transmitting external signals to cause altered gene responses. Although the native *E. coli* QS system is a complex network, it lends itself well for use in synthetic biology because the functional QS units can be parsed and rearranged to serve as synthetic genetic switches, oscillators, or biosensors.[5] Most often, these functional units have been built out of more focused autoinducer circuits or species—specific genetics (i.e., the LuxIR system). Here, the native circuitry of *E. coli* is exploited, and given its reach among bacteria, perhaps *lsr* controllers will find broad based utility. LsrR represents a key point of access for gene expression control in response to QS signals, and thus, it was our focus for discovering novel QS controlled switches through the directed evolution of a QS transcriptional regulator. Our approach was to utilize this technique to create LsrR synthetic switches able to generate novel responses from the *lsr* promoter. Directed evolution mutant eLsrR was found to have enhanced *lsr* promoter repression and greater derepression in response to the QS signal, AI-2, beyond that of the native LsrR. Additionally, mutant aLsrR was found to have reversed repression/derepression function on the *lsr* promoter. That is to say, in the presence of the QS signal, aLsrR represses gene expression and it is derepressed in the absence of the signal molecule. Protein modeling and docking studies were used to further understand the impact of these mutations on AI-2 binding and provide insight into their unique functionality. Additionally, the utility of aLsrR as a sensor was demonstrated through a synthetic ecosystem where it received and responded to a signal from another strain. These new QS-responsive switches represent the first evolved AI-2 regulators, thus expanding the capabilities of AI-2 QS circuitry in synthetic biology.

RESULTS AND DISCUSSION

LsrR Can Be Modified through Directed Evolution. LsrR is the transcriptional repressor of the *lsrRK* and *lsr* operons.[28,32] By evolving this transcriptional regulator, QS-controlled, synthetic biological switches were engineered. Screening LsrR directed evolution mutants for altered expression profiles in several QS mutants was used to identify these switches. A library of altered LsrR genes was created using error prone PCR and were inserted into a *lacZ* reporter plasmid, where the *lsr* promoter controlled *lacZ* expression. The resulting plasmid library was transformed first into *E. coli* QS mutant BA1, (Δ*lsrR, lsrK, lacZ*), followed by QS mutants LW8 (Δ*lsrR, lacZ*) and SH1 (Δ*luxS, lsrR, lacZ*) to evaluate novel modulations of LsrR activity by unphosphorylated AI-2, phospho-AI-2, and absence of AI-2, respectively. LsrR activity was measured by *lacZ* expression from the *lsr* promoter, as shown schematically in Figure 1a. Under native conditions, LsrR binds the *lsr* promoter in the absence of phospho-AI-2 and represses gene expression; resulting in no β-galactosidase detection. However, the presence of phospho-AI-2 causes LsrR derepression, allowing gene expression to resume and β-galactosidase to be produced. The expected β-galactosidase production profile in the presence of a plasmid-encoded native LsrR is shown in Figure 1b. In the Δ*lsrR, lsrK* mutant (BA1), *lacZ* expression is very low because AI-2 is unphosphorylated, resulting in LsrR binding to the *lsr* promoter. Similarly, there is low *lacZ* expression in the Δ*luxS, lsrR* mutant (SH1) because this strain does not synthesize AI-2. Mutant LW8 (Δ*lsrR*) has high β-galactosidase production because LsrR is present on the plasmid and is derepressed from the *lsr* promoter by phosphorylated AI-2 (as this strain contains chromosomal copies of both *luxS* and *lsrK*). When LsrR is absent, gene expression from the *lsr* promoter is high in all three mutants, as there is no repressor present to inhibit β-galactosidase production (Figure 1c).

The directed evolution library was first screened in strain BA1 for isolates exhibiting high levels of *lacZ* expression in the absence of phospho-AI-2. Detection of β-galactosidase in this screening indicated that the LsrR protein was not bound to the promoter. This lack of promoter binding could result from two distinct scenarios: (1) the presence of mutations that prevent direct promoter binding by altering the DNA binding domain, and therefore repression is never possible; (2) mutations that alter the interaction of the protein with the QS signal and repression is possible with the correct signal. To distinguish between these two possibilities, LsrR mutants with high *lacZ* expression in BA1 were also evaluated in two other QS mutant strains: SH1, which lack *luxS* and therefore did not produce any AI-2, and LW8, which produces phospho-AI-2. Evolved LsrR mutants with high *lacZ* expression in all strains were indicative of scenario 1, where the promoter binding ability had been abolished. Such mutants were similar to the negative control strain that lacked any LsrR (chromosomal or plasmid) (Figure 1c) and showed high *lacZ* expression under any circumstances examined.

Reverse Function LsrR. One mutant, aLsrR, was found to have the converse *lacZ* expression profile. In this case, we found high *lacZ* expression in BA1 where there is no kinase, and in SH1, a *luxS* knockout that does not produce AI-2. Taken together, these results might suggest abolished binding commensurate with the set of mutants described in scenario 1. Surprisingly, however, *lacZ* repression was observed in the presence of phosphorylated AI-2 (strain LW8) (Figure 1d). These results suggest that aLsrR represses gene expression from the *lsr* promoter in the presence of phospho-AI-2 but is derepressed by the unphosphorylated AI-2, as well as the absence of AI-2. This is noteworthy because aLsrR putatively operates in the converse manner as the native LsrR. That is, the prevailing understanding is that native LsrR binds DNA in the

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LsrR        MTINDSAISEQGMCEEEQVARIAWFYYHDGLTQSEISDRLGLTRLKVSRLLEKGHQSGII 60
eLsrR       MTINDSAISEQGMCEEEQVARIAWFYYHDGLTQSEISDRLGLTRLKVSRLLEKGHQSGII 60
aLsrR       MTINDSAISEQGMCEEEQVARIAWFYYHDGLTQSEISDRLGLTRLKVSRLLEKGHQSCII 60

LsrR        RVQINSRFEGCLEYETQLRRQFSLQHVRVIPGLADADVGGRLGIGAAHMLMSLLQPQQML 120
eLsrR       RVQINSRFEGCLEYETQLRRQFSLQHVRVIPGLADADVGGRLGIGAAHMLMSLLQPQQML 120
aLsrR       RVQINSRFDGCLEYETQLRRQFSLQHVRVIPGLADADVGGRLGIGAAHMLMSLLQPQQML 120

LsrR        AIGFGEATMNTLQRLSGFISSQQIRLVTLSGGVGSYMTGIGQLNAACSVNIIPAPLRASS 180
eLsrR       AIGFGEATMNTLQRLSGFISSQQIRLVTLSGGVGSYMTGIGQLNAACSVNIIPAPLRASS 180
aLsrR       AIGFGEATMNTLQRLSGFISSQQIRLVTLSGGVGSYMTGIGQLNAACSVNIIPAPLRASS 180

LsrR        ADIARTLKNENCVKDVLLAAQAADVAIVGIGAVSQQDDATIIRSGYISQGEQLMIGRKGA 240
eLsrR       ADIARTLKNENCVKDVLLAAQAADVAIVGIGAVSQQDDATIIRSGYISQGEQLMIGRKGA 240
aLsrR       ADIARTLKNENCVKDVLLAAQAVDVAIVGIGAVSQQDDATIIRSGYISQGEQLMIGRKGA 240

LsrR        VGDILGYFFDAKGDVVTNIKIHNELIGLPLSALKTIPVRVGVAGGENKAEAIAAAMKGGY 300
eLsrR       VGDILGYFFDAKGDVVTNIKIHNELIGLPLSALKTIPVRVGVAGEENKAEAIAAAMKGGY 300
aLsrR       VGDILGYFFDAKGDVVTNIKIHNELIGLPLSALKTIPVRVGVAGGENKAEAIAAAMKGGY 300

LsrR        INALVTDQDTAAAILRS 317
eLsrR       INALVIDQDTAAAILRS 317
aLsrR       INALVTDQDTTAAILRS 317
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Figure 2. Alignment of two evolved LsrRs, eLsrR and aLsrR, with the native repressor. Mutated residues are highlighted in yellow.

absence of AI-2/phospho-AI-2 and is derepressed by phospho-AI-2. aLsrR has the opposite response to both the absence of AI-2 and the presence of unphosphorylated AI-2 as native LsrR. Comparing the *lacZ* expression levels in the presence of phospho-AI-2 in LW8, expression is almost 200-times higher in the derepressed native LsrR as compared to the repressed aLsrR (Figure 1b and d). Conversely, derepressed aLsrR in the absence of AI-2/phospho-AI-2 exhibited a 14-fold and 16-fold increase in expression relative to the native LsrR in SH1 and BA1, respectively. Furthermore, when *lacZ* expressions with both LsrRs are compared in their respective derepression states, expression in the presence of aLsrR is double that of native LsrR. We also noted that *lacZ* expression is also higher with aLsrR in the absence of AI-2 and phospho-AI-2 as compared the negative control (Figure 1c and d).

Sequence analysis of aLsrR revealed four mutations, two in the proposed DNA binding domain (G58C and E69D) and two in the proposed signal binding domain (A203V and A311T) (Figure 2). It is not surprising that the odd nature of aLsrR involved mutations at multiple sites, as many secondary effectors, such as dimerization or other transcriptional factors (e.g., cAMP/CRP) reported to influence *lsr* and *lsrRK* expression,[28,30,32,33] may be involved. That is, the exact role by which aLsrR and LsrR modulate gene expression needs further analysis. In addition to its known function in QS, it likely binds other promoters throughout the *E. coli* genome and its potential role as an activator has been reported.[27] The *Pantoea stewartii* subsp. *stewartii* QS repressor, EsaR, has two known binding sites: the promoter P_{easR}, where it represses gene expression in the absence of an AI molecule and promoter P_{easS}, where it activates gene expression in the absence of an AI molecule. Although no definitive activator DNA binding sites have been identified, it is conceivable that activator sites may exist for LsrR as they do for EasR.

The use of directed evolution to develop improved-function proteins is a common approach, as it is easier to refine existing molecules rather than construct or discover new ones. However, generating a novel function is rare,[34] and examples of this are few. Scholz et al.[35] evolved the Tet repressor for activity reversal; instead of activating gene expression when bound to the inducer, anhydrotetracycline, this led to repression. The authors suggested that the mutations caused a repositioning of the DNA-binding domain leading to inducer-bound conformational change resulted in the opposite activities. Meyers also used directed evolution of the lac repressor, LacI, to generate functionally inverted variants.[36] Although we can only speculate on the effects of the aLsrR mutations on the mechanism of promoter repression and activation, functional "reversal" repressors have been generated and used in other applications.[37−39]

Enhanced LsrR. During the *lsr* expression screening of the evolved LsrR library mutants, another interesting mutant was identified that appeared to have enhanced native LsrR functions. eLsrR was found to be a better repressor of the *lsr* promoter and when provided with phospho-AI-2 (derepressed) yielded more expression than the native LsrR (Figure 1e). In the absence of phospho-AI-2, repression of the *lsr* promoter is naturally leaky,[20,32,40] leading to a background level of the Lsr transporter, LsrR repressor, and LsrK kinase, all of which are up regulated upon AI-2 entry into the cell. There is clear repression of the *lsr* promoter by native LsrR in the absence of phosphorylated AI-2, and eLsrR exhibits superior repression than LsrR. That is, *lacZ* expression was decreased 97% in QS mutant SH1 expressing eLsrR as compared to native LsrR (Figure 1b and e). In addition to enhancing the repressor activity of LsrR, the evolved protein also showed enhanced derepression. That is, there was a 25% increase in *lacZ* expression over native LsrR when cells produced both phosphorylated AI-2 and eLsrR. Sequence analysis of eLsrR revealed that there were two amino acid mutations: G285E and T306I (Figure 2). Both mutations are located in the proposed signal binding domain. We performed computational docking simulations to shed some light on the small molecule binding

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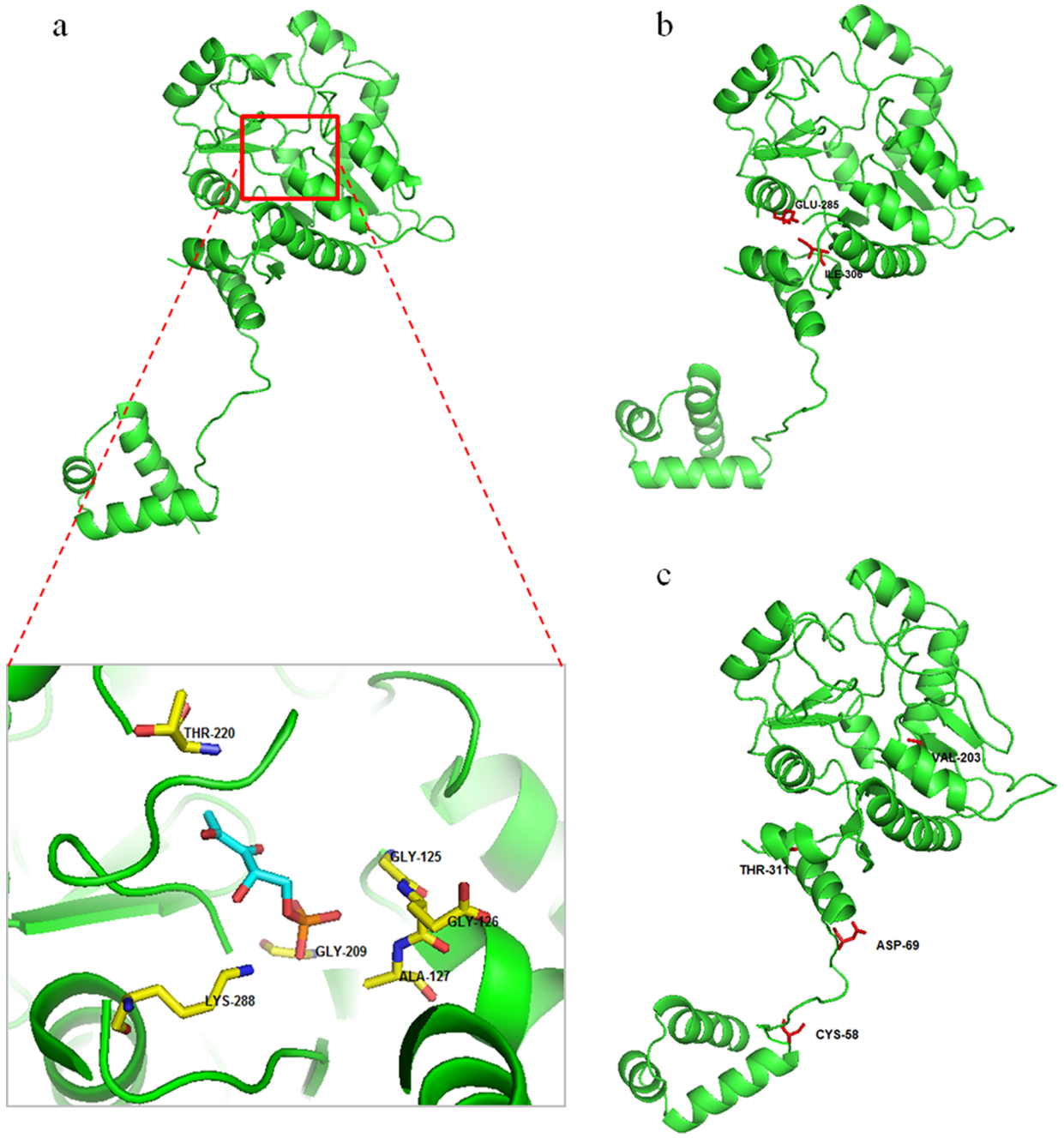

Figure 3. Predicted structure of (a) native LsrR, (b) eLsrR, and (c) aLsrR, using ESyPred3D. Mutated residues are highlighted in red. The insert in panel a shows the putative phospho-AI-2 binding site on LsrR, as identified via docking.

domain and the putative binding of AI-2 and phospho-AI-2, as discussed below.

QS repressors have been previously evolved to expand signal recognition repertoire. Recently, Shong et al.[41] evolved the *P. stewartii* QS repressor, EsaR, to recognize other QS signal molecules as well as its native signal molecule. In addition to EasR variants that respond to alternative QS molecules, they also found variants that were more sensitive to the native QS signal and observed repression at a lower signal concentration. Other non-QS repressors have been enhanced through directed evolution. The *lac* operon repressor was evolved by Daber and Lewis.[42] In this study, a heterodimer (LacI is normally a homodimer) that recognized a distinct mammalian operator half-site was produced that bound more tightly to a chimeric operator than the native LacI, reducing the leakiness and resulting in a more pronounced induced/repressed state. Unlike activators, which recruit RNA polymerase to a promoter and may facilitate the interaction, repressors simply bind to a promoter's operator regions and block RNA polymerase binding.[43,44] This gives the engineering of repressors some degree of inherent flexibility, whereas activators and their binding sites tend to be more rigid.[45] This may explain the increased development of engineered repressors and their use in synthetic systems.

Docking of Phospho-AI-2 into Putative Binding Site in LsrR and Mutants. Using docking software Autodock Vina, we docked phospho-AI-2 into the predicted structure of LsrR and mutants (Figure 3). A putative phospho-AI-2 binding site,

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containing a lysine residue (Lys288), was identified. Typically protonated lysine or arginine residues stabilize phosphate groups so in the case of LsrR, Lys288 is expected to stabilize the phosphate group in phospho-AI-2 via a salt bridge.[46] Most of the mutations in aLsrR and eLsrR seem to be outside the phospho-AI-2 binding site (Figure 3c and d) so we postulate that the effects of the mutations are not due to the abrogation of key residue−ligand interactions in the ligand binding site but probably due to conformational effects on the local structure of the protein caused by the mutations.

QS-Mediated Switch. The ability of cell populations to communicate with one another and initiate behaviors in a programmed manner is critical for carrying out complex synthetic biology functions.[4] We have demonstrated that aLsrR can respond to endogenously produced AI-2 by repressing gene expression from the *lsr* promoter. However, to demonstrate the practical use of aLsrR as a QS-mediated switch, aLsrR should respond to exogenous AI-2, for example, in a synthetic mixed microbial population. We sought to test whether we could alter information exchange among two "communicating" cell populations via a completely new and untested mechanism. Figure 4a conceptually illustrates how this novel signaling mechanism may work using a sender and receiver strain and AI-2 as the communication signal between them. The receiver strain (through aLsrR) has an "on" phenotype when the sender strain produces low levels of extracellular AI-2. The receiver strain alters its phenotype to "off" when the sender strain increases AI-2 production, resulting in increased levels of extracellular AI-2.

We were able to demonstrate this experimentally using LW9 as the sender strain, SH1 paLsrR as the receiver strain and *β*-galactosidase activity as the phenotype. LW9 is a QS mutant that lacks both *lacZ* and the *lsr* operon (*lsrACBDFG*). Therefore, it does not produce *β*-galactosidase or the AI-2 transporter, which results in excessive extracellular AI-2. The receiver strain was SH1, which does not produce AI-2 and contains a plasmid-encoded aLsrR (paLsrR). SH1 paLsrR, in the absence of AI-2, has high *β*-galactosidase production and is repressed by the presence of AI-2. The *β*-galactosidase activity of the sender strain alone, receiver strain alone, and the two in coculture over a 10 h time course in shown in Figure 4b. The sender strain, LW9, alone has almost undetectable levels of *β*-galactosidase activity over the entire time course. This is expected because it lacks the *lacZ* gene. The receiver strain, SH1 paLsrR, alone showed increasing levels of *β*-galactosidase activity over time as the population expanded due to cell growth. In coculture, the *β*-galactosidase activity also increased over time; however it was consistently lower than the levels measured in the receiver only culture.

It is important to note that coculture *β*-galactosidase activity is affected by both the number of receiver cells and the level of gene expression. In order to differentiate between these two factors and calculate the true QS-mediated *β*-galactosidase attenuation, the coculture *β*-galactosidase activity was adjusted to reflect the percentage of the SH1 paLsrR activity. As the growth rates of LW9 and SH1 paLsrR were the same, a 1:1 dilution factor was also taken into account in this adjustment. The *β*-galactosidase activity in the coculture and SH1 paLsrR during the first three hours in culture was low, and there was no significant difference between them. By 4 h in culture, marked differences between the two conditions could be observed and the coculture had approximately 70% of the *β*-galactosidase activity relative to SH1 paLsrR alone throughout the time course (Figure 4c). While less than anticipated, these data indicate that LW9 can send a signal in the form of AI-2, which is perceived by SH1 paLsrR, and SH1 paLsrR then modulated its behavior by reducing *β*-galactosidase production relative to its noncommunicating control. We note that *β*-galactosidase is a very stable enzyme[47] and previously synthesized enzyme is likely to be active even though *lacZ* expression was attenuated by the aLsrR. Therefore, it is likely the actual repression was greater than what was measured. We are currently developing a

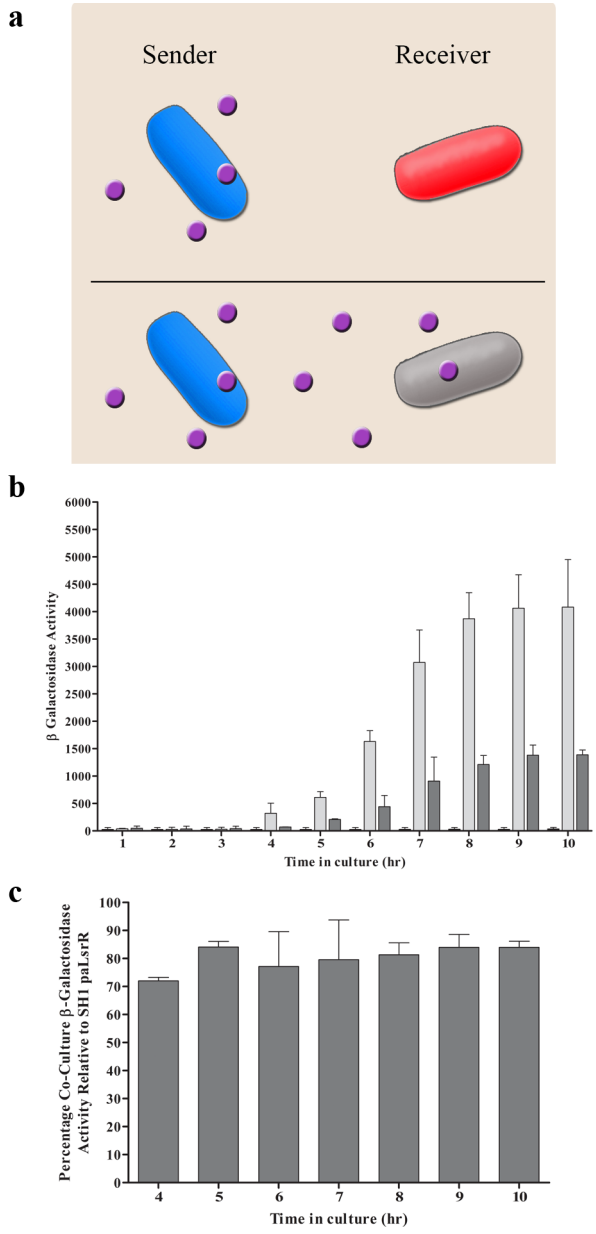

Figure 4. Use of aLsrR as a QS-mediate switch. (a) schematic of sender strain, LW9 (represented in blue), producing the AI-2 signal (represented as purple circles). This is sensed by receiver strain, SH1 paLsrR (represented in red as active). Internalization and processing of the signal results in the repression of gene expression in the receiver strain (represented in gray as inactive). (b) *β*-galactosidase activity in LW9 pure culture (black bars), SH1 paLsrR pure culture (light gray bars), and SH1 paLsrR/LW9 coculture (dark gray bars) over 10 h of culture. (c) Percentage of SH1 paLsrR *β*-galactosidase activity in coculture relative to SH1 paLsrR *β*-galactosidase activity in pure culture.

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set of destabilized reporter constructs to enable a more dynamic analysis. Using this approach, we expect to demonstrate a wider range of functions and applications for these regulators.

Although the coculture experiments described here represent a model example, they also demonstrate that discrete cell populations can be controlled autonomously through altered native QS genetic circuitry. Autonomous control of cell populations has distinct advantages beyond the obvious lack of operator input. They can guide behavior, including that of a subpopulation, based on prevailing conditions or cues.[40,48] More specifically, a sender strain can control the phenotype of a receiver strain using a single, robust signal molecule (AI-2) and synthetic regulators, such as eLsrR and aLsrR. We suggest this as a desired feature of an engineered microbial consortium as it fine-tunes a metabolic process or population-based phenotype. A QS-mediated switch could be used to initiate or halt a given pathway by other cells in the consortia, thus creating a self-regulating population. In our above examples, populations were controlled to express recombinant proteins[40] and swim to and integrate feature density on nearby cell surfaces.[48]

Conclusions. Transcriptional regulators and regulatory regions are common elements of a synthetic biology toolbox.[5] However, there are only a few promoters utilized.[49] By engineering a novel LsrR, the *lsr* promoter can be built upon as yet another member in the toolkit. That is, the generation and characterization of aLsrR and eLsrR has important implications for the creation of new synthetic networks because it allows the *lsr* promoter to be more finely tuned. aLsrR is a novel LsrR variant that has a reverse function and can act as an activator of the *lsr* promoter in the absence of AI-2/phospho-AI-2. As this protein also responds to AI-2, it may be useful, in conjunction with the native repressor, to create a biological signal splitter. When used in combination with eLsrR to control the expression of different genes, AI-2 can act as a single signal input that switches expression of one gene "on" in one population and expression of a different gene "off" in another population (Figure 5). The addition of eLsrR to the toolbox is also advantageous because the natural leakiness of the native QS circuit can be problematic in QS- sensor and detection applications. The use of eLsrR may prove ideal in functions where the AI-2 mediated gene expression must be tightly controlled.

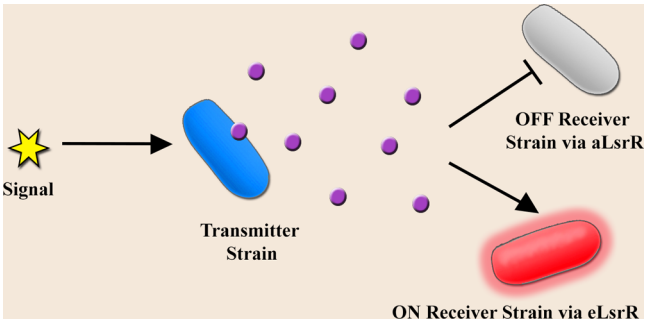

Figure 5. Schematic of a QS-based biological signal switch utilizing evolved LsrR regulators. Two disparate cell population can be controlled simultaneously with a single AI-2 signal molecule. Cell populations possessing positive regulatory eLsrR can up-regulate gene expression in the presence of AI-2, while those possessing negative regulatory aLsrR can concurrently represses gene expression. In this figure, an extracellular signal is detected by a transmitter strain, causing it to become activated and synthesize AI-2 (purple circles). The presence of high AI-2 concentrations is sensed by two different types of receiver strains. The OFF receiver strain halts gene expression upon sensing AI-2 and the ON receiver strain initiates gene expression. Removal of the signal will terminate AI-2 synthesis, and the two receiver strains will reverse their gene expression states.

METHODS

Bacterial Strains and Culture Conditions. Bacterial strains employed in this study are listed in Table 1 and were cultured in LB medium. When necessary, the media were supplemented with the following antibiotics: 50 μg/mL ampicillin; 50 μg/mL kanamycin. BA1, a double chromosomal knockout of *lsrR, lsrK*, was created by a one-step replacement method described by Datsenko and Wanner.[50] Briefly, pKD4 was PCR amplified with primers lsrRHP1 and lsrRHP2[51] and introduced into LW11c pKD46 (expresses the Red recombinase) by electroporation. Recombinants were selected on LB supplemented with kanamycin and plasmid pKD46 was cured by growth at 37 °C. Gene replacement was confirmed by PCR using primers BamHILsrRF, Kan2, LsrRREcoR1, and Kan1, followed by the removal of the resistance gene using a helper plasmid, pFLPe-Tet (Gene Bridges) that expresses the FLP recombinase. Elimination of the kanamycin resistance as confirmed through antibiotic susceptibility and PCR using BamHILsrRF and LsrRREcoR as well as DNA sequencing. The antibiotic resistance genes were similarly removed from LW11, LW8, and SH1 to produce LW11c, LW8c, and SH1c.

Table 1. Bacterial Strains and Plasmids

E. coli strains	description	reference
ZK126	W3110 Δ*lacUl 69-tna2*	57
LW11	ZK126 *lsrK*:Kan	32
LW8	ZK126 *lsrR*:Kan	32
LW9	ZK126 *lsr* operon:Kan	32
SH1	LW8 *luxS:Cm*	58
BA1	LW11 Δ*lsrR*	this study
LW11c	LW11 ΔKan	this study
LW8c	LW8 ΔKan	this study
SH1c	SH1 Δ*Cm*	this study
plasmid	description	reference
pCT1	pFZY1 derivative, *lsr* intergenic region fused to *lacZYA*	40
pCT1-lsrR	pCT1 derivative with native *lsrR* inserted upstream *lsr* intergenic region	this study
peLsrR	pCT1 derivative with eLsrR inserted upstream *lsr* intergenic region	this study
paLsrR	pCT1 derivative with aLsrR inserted upstream *lsr* intergenic region	this study

LsrR Directed Evolution and Reporter Plasmid Construction. The *lsrR* gene was amplified from *E. coli* ZK126 genomic DNA using primers described in Table 1 using GeneMorph II Random Mutagenesis kit according to manufacturer instructions (Strategene) for a mutation rate 2–7 nucleotide changes per gene. The PCR products were gel purified and digested with *Eco*RI and *Bam*HI. Plasmid pCT1 (Table 1), which is a single copy *lsr* promoter driven *lacZ* reporter plasmid, was also digested with *Eco*RI and *Bam*HI. The

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Table 2. Primers

name	sequence	description
lsrRF	CAAAGTGAAGAATGAATTATGACA	upstream primer for genomic lsrR
lsrRR	ATCATTCCCGGTAATAAGGTC	downstream primer for genomic lsrR
KanF	CAGTCATAGCCGAATAGCCT	upstream primer for kanamycin resistance gene
KanR	CGGTGCCCTGAATGAACTGC	downstream primer for kanamycin resistance gene
BamHILsrRF	TGAATTGGATCCATGACAATCAACGATTC	upstream primer for directed evolution lsrR with *Bam*HI digestion site
LsrRREcoRI	CCGGAATTCTCTTAACTACGTAAAATCGC	downstream primer for directed evolution lsrR with *Eco*RI digestion site
ForSeq4	GGCCCTTTCGTCTTTAAG	upstream vector primer for lsrR sequencing
RevSeq1	CCCTATGTTCAAAGTGAAGAATG	downstream vector primer for lsrR sequencing

library of PCR fragments were ligated into digested pCT1 using Quick ligase (New England Biolabs) and transformed into BA1. Transformants were plated on LB agar supplemented with ampicillin and Xgal. pCT1-lsrR was similarly created using a high-fidelity polymerase to amplify *lsrR* with the same primers and inserted into pCT1 by the same restriction enzymes sites.

Lsr Operon Expression. Overnight cultures were diluted 1:100 into fresh LB with ampicillin and grown at 37 °C, 250 rpm to an OD600 0.8−1.0. A culture volume of 250 μL was collected by centrifugation at 10 000 × g for 5 min and resuspended in 500 μL PBS for 2 h at 37 °C. Quorum sensing dependent β-galactosidase production was quantified by the Miller assay.[52] For coculture experiments, overnight cultures were diluted into fresh LB with ampicillin to a starting OD600 0.01 and grown at 37 °C, 250 rpm. A culture volume was collected at the indicated time points and β-galactosidase production was quantified by the Miller assay.[52]

LsrR Sequencing and Predicted Structure Modeling. Plasmids peLsrR and paLsrR were purified from BA1 and the *lsrR* gene was amplified using primers ForSeq4 and RevSeq1 (Table 2). The PCR product was gel extracted and sequenced by the Institute of Bioscience and Biotechnology Research DNA sequencing facility (UMD College Park). The DNA sequences of the three *lsrR* genes were compared to native *lsrR*, mutated base pairs were identified, and the sequences were translated (Expasy) to yield the protein sequences.

Docking calculations were performed using Autodock Vina 1.1.1.[53] A grid box large enough to cover the ligand binding pocket was chosen. The exhaustiveness value was set as 32 in the Autodock calculations and the rest of the parameters were used as default. All rotatable bonds in the ligand were set as freely rotatable, with the exception of the C−C bond between the two carbonyls. The receptor protein was considered rigid. 3D structures were predicted by ESyPred3D,[54] and the ligand PDB file was prepared by ChemDraw. Autodock Tools 1.5.4[55] was used to convert the PDB files into PDBPT files for the Autodock Vina calculations. For each binding site, only the orientation of the ligand with the lowest binding affinity was considered as the predicted ligand bound form to the receptor. Binding sites with an affinity greater than −4.8 kcal/mol were deemed not to have a great affinity for the ligands. 2D structures were predicted with PSIPRED.[56]

■ ASSOCIATED CONTENT

Supporting Information

Schematic showing the *E. coli* quorum sensing circuit. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

QS, quorum sensing; AI, autoinducer; AI-2, autoinducer-2; phospho-AI-2, phosphorylated autoinducer-2

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