Rational design of ‘controller cells’ to manipulate protein and phenotype expression

**Amin Zargar^{1,2}, David N. Quan^{1,2}, Milad Emamian^{1,2}, Chen Yu Tsao^{1,2}, Hsuan-Chen Wu^{1,2},
Chelsea R. Virgile^{1,2}, William E. Bentley^{1,2*}**

¹Institute for Bioscience and Biotechnology Research (IBBR)

University of Maryland

College Park, MD 20742, USA

²Fischell Department of Bioengineering

University of Maryland

College Park, MD 20742, USA

***William E Bentley**

2330 Jeong H. Kim Engineering Building,

College Park, MD, 20742

bentley@umd.edu

ABSTRACT

Coordination between cell populations via prevailing metabolic cues has been noted as a promising approach to connect synthetic devices and drive phenotypic or product outcomes. However, there has been little progress in developing ‘controller cells’ to modulate metabolic cues and guide these systems. In this work, we developed ‘controller cells’ that manipulate the molecular connection between cells by modulating the bacterial signal molecule, autoinducer-2, that is secreted as a quorum sensing (QS) signal by many bacterial species. Specifically, we have engineered *E. coli* to overexpress components responsible for autoinducer uptake (*lsrACDB*), phosphorylation (*lsrK*), and degradation (*lsrFG*), thereby attenuating cell-cell communication among populations. Further, we developed a simple mathematical model that recapitulates experimental data and characterizes the dynamic balance among the various uptake mechanisms. This study revealed two controller “knobs” that serve to increase AI-2 uptake: overexpression of the AI-2 transporter, LsrACDB, which controls removal of extracellular AI-2, and overexpression of the AI-2 kinase, LsrK, which increases the net uptake rate by limiting secretion of AI-2 back into the extracellular environment. We find that the overexpression of *lsrACDBFG* results in an extraordinarily high AI-2 uptake rate that is capable of completely silencing QS-mediated gene expression among wild-type cells. We demonstrate utility by modulating naturally occurring processes of chemotaxis and biofilm formation. We envision that ‘controller cells’ that modulate bacterial behavior by manipulating molecular communication, will find use in a variety of applications, particularly those employing natural or synthetic bacterial consortia.

KEYWORDS

Quorum sensing; chemotaxis; biofilm; autoinducer 2; quorum quenching

1. INTRODUCTION

Metabolic engineering exploits the genetic modification of cellular pathways to improve production of metabolites and proteins [1, 2]. Many noteworthy examples have been demonstrated wherein these cells serve as ‘factories’ for the environmentally sustainable production of energy, materials, and chemicals [3]. Towards this aim, metabolic engineering has incorporated finely tuned synthetic controllers and cells in the creation of artificial networks [4-8]. The general structure of these synthetic networks is based on control devices that respond to specific stimuli in a predictable fashion [9, 10]. However, the task of coordinating among and between cell populations remains a critical challenge that can limit the production of desired end-products [11, 12]. A further challenge is controlling the partitioning of resources that (i) maintain native metabolism and (ii) adequately support product synthesis [13, 14]. One creative approach to address both of these challenges is through the leveraging of cell-cell communication networks, and these have been the target of a variety of dynamic control systems [15-17]. Using the native bacterial signaling network known as quorum sensing [18, 19], we have previously shown the ability to reduce the metabolic burden [20, 21] and “program” cell populations through the metabolic cue, autoinducer-2 [22, 23]. However, while there has been much development in multicellular systems that respond to metabolic cues [24-27], control of the intensity of these metabolic cues to fulfill the potential of spatiotemporal control has not been realized.

In this work, we have developed bacterial AI-2 consumers, ‘controller cells’, which can be deployed to control AI-2 in a predictable fashion using the now well-characterized quorum sensing mechanisms of *E. coli* (**Scheme 1A**). AI-2 is synthesized and recognized by a wide variety of bacteria [28, 29]; correspondingly its use as a potential target for modulating QS activities among different cell types is of interest. The use of genetically engineered bacteria to ‘quench’ extracellular AI-2 was first described by Xavier et al. [30], where genetic deletions of its synthase (*luxS*) and its repressor (*lsrR*) were used to interfere with bacterial communication. However, the interrelated complexity of QS networks renders the elucidation of its mechanisms difficult, and the production of simple, “modular” networks would enrich the understanding of these actions [31]. We have addressed this through the model-based design, construction, and characterization of these ‘controller cells’ to modulate the external AI-2 environment. These cells are designed via the compartmentalization of different aspects of AI-2 processing: uptake (*lsrACDB*), phosphorylation (*lsrK*), and degradation (*lsrFG*) (**Scheme 1B-1C**).

These ‘controller cells’ provide the ability to regulate extracellular AI-2 and modulate synthetic circuits. Further, we show that the ability to quench extracellular AI-2 through ‘controller cells’ can attenuate the native cell-cell behaviors of chemotaxis and biofilm formation. By teasing apart the regulatory network for AI-2, we have enhanced our understanding of the collective population-scale response to AI-2. In this way, systems can be designed wherein we decouple the consumption of AI-2 from bacterial population density and its emergent behavior. With the addition of ‘controller cells’, we provide an orthogonal means to modulate QS activity, demonstrate their use as mediators of heterologous protein and phenotype expression, and provide a modeling foundation to guide QS-communication.

2. MATERIALS AND METHODS

2.1. Plasmid construction

The bacterial strains and plasmids used in this study are listed in **Table S1**, and were constructed according to standard procedures [32]. Briefly, plasmid pTrcHisB (Invitrogen) was used as the backbone to construct plasmids pLsrFG, pLsrK, pLsrACDB, and pLsrACDBFG. The sequences for *lsrFG*, *lsrK*, *lsrACDB*, and *lsrACDBFG* were amplified by PCR using Q5 polymerase (New England Biolabs) from *E. coli* K-12 strain W3110. These PCR inserts were ligated into XhoI-digested pTrcHisB using Gibson assembly [33] and then transformed into LW12 (W3110 $\Delta luxS$) [34]. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in **Table S2**. Cloning was verified with sequencing and Western Blot.

2.2. AI-2 assay

Cultured media was tested for the presence of AI-2 by inducing luminescence in *Vibrio harveyi* reporter strain BB170 [35]. Briefly, BB170 was grown for 16 hours with shaking at 30°C in AB (AI-2 Bioassay) media. AB media is made by adjusting 400 mL of distilled (DI) water to pH 7.5, and adding 7 grams of NaCl, 2.4 grams of MgSO₄, 0.8 grams casamino acid, and 8 mL of glycerol. AB media is supplemented with 400 μ L of potassium phosphate buffer (K₂HPO₄ 10.71g and 5.24g KH₂PO₄ in 100 mL of DI water), 400 μ L of 0.1M L-arginine (0.1742g in 10 mL of DI water), 40 μ L of riboflavin (10 μ g/mL), 40 μ L of thiamine (1 mg/mL) and 40 μ L kanamycin (50 mg/mL).

Overnight cultures were diluted 1:5,000 in fresh AB media with kanamycin, and aliquoted into sterile 12 x 75-mm tubes (Fisher Scientific). Test samples were added to BB170

cultures at a final concentration of 10% (vol/vol). Luminescence was measured by quantifying light production with a luminometer (EG&G Berthold LB 9509 Jr) and assays were adjusted, if needed, so that values were in the linear range. Data are presented as “fold change” compared to negative controls. All conditions were tested in triplicate. In experiments with supplemented chemically-synthesized AI-2, we report AI-2 activity normalized to the initial concentration, as our previous study showed a linear correlation between AI-2 concentration and resultant bioluminescent AI-2 activity [36].

2.3. AI-2 uptake profiles of ‘controller cells’

Chemically synthesized AI-2 [37] was generously provided by the Sintim research group. Each strain was reinoculated by diluting an overnight culture to 3% volume in 10 mL of LB; these cells were grown in a 50 mL culture flask to an optical density (OD) ~ 0.4-0.6 at 30°C with 250 RPM shaking. Isopropyl β -D-1-thiogalactopyranoside (IPTG) and AI-2 were added to a final concentration of 1 mM and 50 μ M, respectively. Every half hour, optical density was measured and samples were harvested for analysis. The average bioluminescence for samples at $t = 0$ was denoted 50 μ M, and subsequent AI-2 activity values were normalized to this concentration.

2.4. Modulation of AI-2 in co-cultures

BL21 pTrcHisB, LW12 pTrcHisB, and LW12 pLsrACDBFG were reinoculated at 3% of overnight culture in 25 mL of LB in 125 mL culture flasks and grown to an OD ~ 0.4-0.6 at 37°C with ampicillin. Co-cultures of BL21 pTrcHisB incubated with either LW12 pTrcHisB or LW12 pLsrACDBFG were aliquoted in culture test tubes at ratios of 9:1, 3:1, 1:1, 1:3, and 1:9. IPTG (1 mM) was added and every 30 minutes optical density was measured and samples were harvested, on which AI-2 activity assays were performed.

2.5. Silencing of autoinduced protein expression

LW12 pTrcHisB and LW12 pLsrACDBFG were reinoculated at 3% overnight culture into 25 mL of LB in 125 mL flasks and grown to an OD ~ 0.4-0.6. The samples were induced with IPTG (1 mM) for three hours, before being resuspended (2000 RPM for 10 minutes) to an OD ~ 1. W3110 pCT6 pET-GFP_{uv} [22], a strain of *E.coli* that responds to the level of the AI-2 concentration by expressing GFP, was grown to an OD ~ 0.2, and then incubated with LW12 pTrcHisB or LW12 pLsrACDBFG. Flow cytometric analysis was performed using a FACSCanto II™ Flow Cytometer (Becton Dickinson) and all raw data was analyzed with BD FACSDiva™ 6.0 software (Becton Dickinson).

2.6. Biofilm studies and evaluation

W3110 pTrcHisB, LW12 pTrcHisB, and LW12 pLsrACDBFG were diluted to OD ~ 0.05 and reinoculated at a total volume of 200 µL at a 1:1 (v/v) ratio. IPTG (1 mM) was added at OD ~ 0.4, and biofilms were cultured for ~ 24 hours (+/- 30 minutes) at 30°C in static conditions. After incubation, optical density was read on a plate reader (Molecular Devices SpectraMax M2) at 600 nm. The supernatant was gently decanted, and each well was washed 3 times with 300 µL of sterile PBS to detach loosely adhered cells. The plate was then incubated at 60°C with the lid off for 60 minutes, and afterwards, 250 µL of 0.1% crystal violet was added to each well and incubated for 15 minutes at room temperature. Crystal violet stain was aspirated with a pipette and excess stain was washed off by gently submerging and mixing in a tray filled with distilled water until washings were free of the stain. After the microplate was air-dried, the dye was resolubilized by adding 250 µL of 95% ethanol, and incubated at room temperature with shaking for 30 minutes. The optical density of each well stained with crystal violet was measured at 540 nm.

2.7. Chemotaxis studies and assay

Preparation of conditioned media: LW12 pTrcHisB and LW12 pLsrACDBFG were inoculated from frozen cell stock in 20 mL of LB with ampicillin in a 125 mL culture flask and grown overnight to OD ~ 0.4 at 23 °C with 150 RPM shaking. The cultures were induced with IPTG (1 mM) for two hours (23 °C, 150 RPM shaking) before being washed and resuspended with DPBS (with calcium and magnesium) to OD ~ 0.4. LW12 pTrcHisB and LW12 pLsrACDBFG cultures were incubated at 37 °C with 250 RPM shaking with 0 µM and 20 µM AI-2. The cell cultures were spun down, and the supernatant (“conditioned media”) was syringe-filtered and stored at -20 °C.

Transwell chemotaxis assay: CT104 pCT6 pET-dsRed cultures [38] were inoculated from frozen stock into 30 mL of LB in 250 mL culture flasks and grown overnight to an OD ~ 0.4-0.6 at 23 °C with 150 RPM shaking. The cells were spun down at 1500 RPM with a fixed rotor for 15 minutes and washed twice with DPBS (supplemented with calcium and magnesium) to an optical density ~ 0.4-0.6. A 3.0 µm transwell was placed in four wells of a 6-well plate (Corning). CT104 pCT6 pET-dsRed cells were first pipetted into the bottom of the wells with a volume of 2.5 mL per well, followed by 1.5 mL of each of the “conditioned media” fluids added to the top of the transwell. The plate was incubated at 30 °C for three hours; cells accumulating in the upper transwell had swum vertically [39]. This method yields fewer motile cells than the reverse scenario (swimming down), but precludes settling in negative controls. The optical density of each sample from the top chamber of the transwell was measured at 600 nm. The experiment was repeated in triplicate.

3. RESULTS

3.1 Design of modular QS elements

As illustrated in **Scheme 1A**, the three steps involved in the processing of AI-2 from the extracellular environment are (i) uptake, primarily through the LsrACDB transporter [34, 40, 41], (ii) LsrK-mediated phosphorylation of AI-2 (to AI-2P), which blocks export back to the extracellular milieu so that accumulated AI-2P binds the regulatory protein LsrR [42, 43], derepressing the Lsr transporter as well as enzymes, LsrF and LsrG, and (iii) degradation of AI-2P through the two step process from isomerase LsrG followed with cleaving and thiolation by LsrF [43, 44]. In this study, we cloned *lsrFG*, *lsrK*, *lsrACDB*, and *lsrACDBFG* into the plasmid pTrcHisB to enable overexpression of all proteins associated with these AI-2 processing mechanisms (**Scheme 1B-1C**). We subsequently transformed each plasmid into LW12, a *luxS* null mutant that cannot synthesize AI-2.

We first characterized the uptake rate of AI-2 by adding a fixed amount of exogenous AI-2 and monitoring the extracellular concentration. Each strain was grown to mid-logarithmic phase (OD ~ 0.4) with the subsequent addition of 50 μ M AI-2 and 1 mM IPTG (see Methods) and optical density was recorded throughout (**Supplementary Figure 1**). **Figure 1A** shows the uptake profile of each strain (colored dots), as well as the results of a topologically simple mathematical model (black trendlines) comprised of several ordinary differential equations (**Table 1**) for state variables AI-2, the molecular species that contribute to AI-2 uptake, and optical density for each cell type. Note, we have included an empty vector control LW12 pTrcHisB that will consume AI-2 ($\Delta luxS$), but not in an accelerated fashion. We found that overexpression of *lsrACDB* and *lsrACDBFG* genes showed the highest rate of uptake, indicating that AI-2 transport into the cell is the slowest step involved in the processing of extracellular AI-

2 into phosphorylated intracellular AI-2. Further, overexpression of *lsrK* resulted in an extracellular AI-2 removal rate slower than overexpression of the transporter, but still faster than the host strain, LW12, with an empty vector or pLsrFG.

Using the described network architecture, our deterministic model yielded simulation results that closely matched the experimental data (**Figure 1A**). The simulated values of AI-2 uptake for each ‘controller cell’ is illustrated in **Figure 1B** and these indicate a broad distribution in the rate of AI-2 uptake through the overexpression of the various uptake mechanisms, and also enrich our understanding of the kinetic balances basis for these phenomena.

In all cases, this is a phenomenological “best fit” model that incorporates the molecular features contributing to uptake, and it is a simplification of our previous stochastic model for AI-2 uptake [45] and deterministic model for *lsr* gene expression [40]. The AI-2 transport into the cell is described as an interaction of the protein complex LsrACDB with extracellular AI-2, and the phosphorylation of AI-2 to AI-2P (a sequestered form of AI-2 that cannot be secreted back outside the cell) is dependent on the interaction of enzyme LsrK with intracellular AI-2. The induction parameter $IPTG_x$ is used as an input (1 or 0) to specify if a plasmid-encoded protein is overexpressed (e.g. pLsrACDBFG has $IPTG_{ACDB}$ value of 1, and $IPTG_K$ value of 0) in the presence of IPTG (strains without induction showed reduced AI-2 uptake, **Supplementary Figure 2**). Our prior work on *lsr* gene expression and AI-2 synthesis [40, 45] provided an initial range of kinetic parameters; a parameter estimation routine was used to fit the model based on a least squares minimization of the distance between the experimental and modeled data (**Table 2**). A detailed discussion of rate equations, growth rates, and kinetic parameters can be found in the **Supplementary material**.

3.2 Quenching of QS-dependent protein expression

We sought to “shut off” W3110 pCT6 pET-GFP_{uv}, a strain that produces and responds to AI-2 by producing GFP, to provide an independent means to alter heterologous gene expression [22]. In **Figure 2**, we show that a 1:1 mixture of W3110 pCT6 pET-GFP_{uv} with LW12 pLsrACDBFG almost completely suppresses QS-activated gene expression from the WT (AI-2 producing) cells for 10 hours. This is in stark contrast to the empty vector results, which found that over 50% of the total population was observed to synthesize GFP over the same time period. Intermediate timepoints show a steady rise in the production of GFP in co-incubations with the empty vector, while co-incubations with LW12 pLsrACDBFG remained low throughout (**Supplementary Figures S3-S4**). “Since both the ‘controller cells’ and W3110 pCT6 pET-EGFP have the same antibiotic resistance, relative population dynamics could not be determined throughout the incubation. However, since LW12 pTrcHisB has a faster growth rate than LW12 pLsrACDBFG (**Table 2**), purely growth rate dynamics would favor decreased fluorescence in cultures of LW12 pTrcHisB than LW12 pLsrACDBFG. Because we chose the ‘controller cell’ strain having the greatest uptake rate, LW12 pLsrACDBFG, we would expect that by either using a lower inoculum fraction or by selecting LW12 pLsrK (the strain that significantly reduced AI-2 levels through overexpression of the kinase) at 50% inoculum we would observe gradations in the overall fraction of QS positive activity. These results demonstrate “programmed” attenuation of heterologous protein expression as an indicator of QS phenotype and typically this is an outcome we seek to maximize. However, such control of the metabolic cue may also have positive implications for guiding synthetic networks of small populations of cells assembled to coordinate to produce a desired outcome [12].

3.3 Manipulation of ‘producer cell’ in co-cultures and extension of model

Previously, we illustrated that the *E. coli* strain, BL21 *luxS*⁺, can act as ‘producer cell’ to increase protein expression in QS-dependent systems [22]. To enable the use of these cells to dynamically modulate metabolic cues, we investigated the interaction of the most effective ‘controller cell’, pLsrACDBFG, and the empty vector with strain BL21 *luxS*⁺. We note that BL21 does not take up AI-2 from the medium [46], hence the removal of AI-2 here is due solely to the added $\Delta luxS$ ‘controller cells’. In this experiment, BL21 pTrcHisB, LW12 pTrcHisB, and LW12 pLsrACDBFG were grown to an OD ~ 0.4 and co-cultures of BL21 pTrcHisB were incubated with either LW12 pTrcHisB or LW12 pLsrACDBFG and aliquoted in culture test tubes at ratios of 9:1, 3:1, 1:1, 1:3, and 1:9. At this point, IPTG (1 mM) was added and samples were harvested every 30 minutes on which AI-2 activity assays were performed and optical density was measured (**Supplementary Figure 5**).

The data in Figure 3A depict the AI-2 levels over time for each initial condition (the initial ratio of BL21 to ‘controller cell’ ranging from 9:1 to 1:9). The initial level of AI-2 is due to wild-type BL21 in the inoculums (they had secreted AI-2 in precultures). As expected, cultures with more BL21 initially had higher AI-2 activity levels and remained highest throughout the incubation. Also, we note that for the control culture, LW12 pTrcHisB (Figure 3A blue bars), there was an appreciable consumption of AI-2 so that we did not find a consistent threefold decrease in AI-2 as the population shifted in threefold increments from 9:1 to 1:9. Nonetheless, a nearly linear decrease was observed with increasing LW12 pTrcHisB cells and this would be expected. In contrast, the rapid uptake rate of LW12 pLsrACDBFG is perhaps most evident in cultures where the consumer culture strain was present at an initial ratio of 1:1 or higher. In these cases,

LW12 pLsrACDBFG (Figure 3A red bars), prevented significant quantities of AI-2 from accumulating to even measurable levels in the extracellular environment throughout the time period, while the empty vector control showed increased accumulation of AI-2 over time at all ratios. Hence, the co-cultured LW12 pLsrACDBFG cells effectively cleared all QS signaling among the ‘producer’ cells at these ratios.

The mathematical model developed in Table 1 was extended to characterize these co-cultures (Figure 3B), as opposed to the addition of exogenous AI-2 to the earlier simulations where AI-2 was exogenously added to ‘controller cell’ cultures (Figure 1). We found good agreement between the model and the experimental data, and detailed discussion of all rate equations, growth rates, and kinetic parameters is provided in the Supplementary material. We note all rate equations and kinetic parameters used in the uptake profile for exogenously added AI-2 were unchanged for the co-incubation experiments.

3.4 Chemotaxis and biofilm attenuation

While this methodology provides a tool to regulate synthetic systems, it may also serve as a modifier of natural processes such as antibiotic susceptibility, motility, and biofilm formation. It has been shown that AI-2 has a contributing effect on biofilm formation in the *E. coli* W3110 [47]; therefore, we investigated if LW12 pLsrACDBFG, could interfere with biofilm formation from this biofilm producer. Co-incubations of W3110 pTrcHisB were reinoculated with either LW12 pTrcHisB or LW12 pLsrACDBFG and grown in a 96-well plate for 24 hours. In certain wells, exogenous AI-2 was added to produce greater biofilm formation, and homocysteine, a side-product of AI-2 synthesis, was added as an additional negative control. Optical density

measurements showed no significant variation in growth (**Figure 4A**), thereby suggesting that this method to obtain biofilm reduction does not exert selective pressure. Biofilm production was normalized to the final cell density and LW12 pLsrACDBFG showed reduced biofilm formation by about 20% compared to identical incubations with LW12 pTrcHisB (**Figure 4B**). A two-tailed unpaired Student's t-test was performed between the groupings, and a p-value of 0.0000485 was determined, indicating that the biofilm reduction is significant. Further, the addition of exogenous AI-2 to the co-incubations (bar 'D') showed a restoration of biofilm formation in co-culture of LW12 pLsrACDBFG while homocysteine (bar 'E') does not, which further illustrates that the direct removal of AI-2 by LW12 pLsrACDBFG caused the resultant biofilm reduction.

Similarly, AI-2 acts as a chemoattractant for *E. coli* [48], and we show that LW12 pLsrACDBFG can interfere with chemotaxis through the removal of AI-2. Conditioned media with exogenously added AI-2 incubated with LW12 pTrcHisB or LW12 pLsrACDBFG was used in a transwell assay where a culture of "seeker" cells, W3110 *ΔluxS ΔlsrFG*, in the bottom chamber traverse the permeable membrane towards the conditioned media with AI-2 in the top chamber (see Methods). As shown in **Figure 5**, a greater population of "seeker" cells chemotaxed upwards towards conditioned media from incubations with LW12 and the empty vector supplemented with AI-2 than LW12 pLsrACDBFG supplemented with AI-2, illustrating that the clearing of AI-2 by LW12 pLsrACDBFG can block AI-2 mediated chemotaxis.

4. DISCUSSION

Connecting both natural and synthetic networks, quorum sensing is widely used for a variety of processes and applications. When we developed the first population based example of a native

regulatory circuit that was rewired to sense and transduce AI-2 to produce proteins, we looked forward to its potential to be used for “throttled” protein expression[22]. This dynamic control could help lead to the development of a ‘synthetic switchboard’ to control multiple genes in industrial bioprocesses [49], and the ‘universal’ signaling molecule AI-2, could be a natural choice to guide this kind of consortia. Traditional metabolic engineering could also benefit from microbial consortia, where a division of labor between two communities decreases the net metabolic load, creates new compounds, and increases productivity [50]. Synthetic communication systems would provide population-level coordination [51], and by developing an orthogonal method to control these QS systems, we provide another useful tool to guide microbial consortia in metabolic engineering applications.

We have previously demonstrated that both native [34] and artificial transcriptional circuits [22, 23] are dependent on the level of signal and that this potentially has widespread application [52-54]. In this work, we developed ‘controller cells, the application of which can actively modulate extracellular AI-2 concentrations. We show that the most effective ‘controller cell’, LW12 pLsrACDBFG, can silence heterologous gene expression. Also, this ‘controller cell’ can independently remove the signal generated from a ‘producer cell’, BL21, revealing the possibility of dynamic modulation (i.e. up- and down-regulation). We extended the application from synthetic networks to the naturally occurring processes of biofilm formation and chemotaxis.

Further, the deterministic model developed here helps to delineate the mechanistic underpinnings that guide QS phenomena. While the increased rate of AI-2 uptake due to LsrACDB overexpression was expected, increased uptake coincident with LsrK overexpression was not (**Figure 1B**). By examining our model results in the context of the roles of the various

components, we hypothesize that enhanced AI-2 uptake was due to a combination of two related factors. The first was that phosphorylated AI-2, unlike AI-2, cannot be transported across the bacterial membrane [55]. Thus, increased LsrK converts more intracellular AI-2 into AI-2P, increasing net AI-2 influx into the cell. Second, phosphorylated AI-2 acts to derepress LsrR, consequently resulting in greater expression of the *lsr*-operon from the genome (that is, AI-2P is assumed to be in rapid equilibrium with LsrR and this level corresponds to the prevailing rate of LsrK expression). So, faster accumulation of AI-2P should result in faster activation of *lsr* – mediated components. Of these two factors, we believe that the dominant is that the increased AI-2P prevents AI-2 from leaking back out of the cell. This was supported by the uptake profile of cells with pLsrFG, which was not significantly different than the uptake profile of cells with the empty vector. Theoretically, overexpression of *lsrFG* should result in faster degradation of AI-2P, which should cause a concomitant decreased expression of the genomic *lsr*-operon and resultantly, a slower uptake of AI-2. Since the uptake rate was not significantly altered, which suggests sufficient AI-2P levels in LW12 pLsrFG to maintain transcription of the genomic *lsr*-operon, then increased levels of AI-2P in LW12 pLsrK from overexpression of LsrK was correspondingly unlikely to cause much greater activation of the genomic *lsr*-operon.

While we have only simulated conditions that describe the experiments shown, its use as a predictive tool for further work is envisioned. That is, the intentional modulation of quorum-sensing molecules by the inclusion of static or growing ‘controller cells’ can provide an additional level of control for synthetic networks. Further understanding of the kinetic parameters of the uptake system would guide future manipulation using this control methodology; one might estimate the quantities of ‘controller cells’ needed to guide protein expression or other processes. Alternatively, we suggest that similarly engineered commensal *E.*

coli may provide a means for altering behavior in natural ecosystems such as the gut microbiome. Analogously, by extension, other bacterial species similarly engineered may provide a means to alter the balance of native niches. To our knowledge these concepts have not been reported. Among the many phenotypes controlled by AI-2 [56] at least two have been demonstrated in *E. coli* [47, 57], and we have shown here that LW12 pLsrACDBFG can guide these phenotypes (biofilm formation and chemotaxis) without exerting selective pressure. We envision such cells might find utility in minimally disturbing cell-cell processes. We recognize that the current system uses a “charged” bacterium (induced with IPTG to overexpress transporter); the advantage being a well-controlled ‘controller cell’. There may be instances where a completely autonomous system would be more advantageous; it might interrogate and interact within ecological niches. While challenges clearly remain for tailoring metabolic cues to spatiotemporally control cell populations, this work provides one additional potent tool for guiding phenotype among bacterial populations.

ACKNOWLEDGEMENTS

Funding provided by the Defense Threat Reduction Agency (DTRA, HDTRA1-13-1-00037), the Office of Naval Research (N000141010446), the National Science Foundation (CBET 1160005, CBET 1264509), and the R. W. Deutsch Foundation.

REFERENCES

1. Bailey, J.E., *Toward a science of metabolic engineering*. Science, 1991. **252**(5013): p. 1668-1675.
2. Stephanopoulos, G. and J.J. Vallino, *Network rigidity and metabolic engineering in metabolite overproduction*. Science(Washington), 1991. **252**(5013): p. 1675-1681.
3. Jarboe, L.R., et al., *Metabolic engineering for production of biorenewable fuels and chemicals: contributions of synthetic biology*. BioMed Research International, 2010. **2010**.
4. Stephanopoulos, G. and J. Kelleher, *How to make a superior cell*. Science, 2001. **292**(5524): p. 2024-2025.
5. Kramer, B.P., M. Fischer, and M. Fussenegger, *Semi-synthetic mammalian gene regulatory networks*. Metabolic engineering, 2005. **7**(4): p. 241-250.
6. Malphettes, L. and M. Fussenegger, *Impact of RNA interference on gene networks*. Metabolic engineering, 2006. **8**(6): p. 672-683.
7. Boyle, P.M. and P.A. Silver, *Parts plus pipes: synthetic biology approaches to metabolic engineering*. Metabolic engineering, 2012. **14**(3): p. 223-232.
8. Keasling, J.D., *Synthetic biology and the development of tools for metabolic engineering*. Metabolic engineering, 2012. **14**(3): p. 189-195.
9. Weber, W. and M. Fussenegger, *Molecular diversity—the toolbox for synthetic gene switches and networks*. Current opinion in chemical biology, 2011. **15**(3): p. 414-420.
10. Purnick, P.E. and R. Weiss, *The second wave of synthetic biology: from modules to systems*. Nature reviews Molecular cell biology, 2009. **10**(6): p. 410-422.
11. Nielsen, J., et al., *Engineering synergy in biotechnology*. Nature chemical biology, 2014. **10**(5): p. 319-322.
12. Cameron, D.E., C.J. Bashor, and J.J. Collins, *A brief history of synthetic biology*. Nature Reviews Microbiology, 2014. **12**(5): p. 381-390.
13. Way, J.C., et al., *Integrating Biological Redesign: Where Synthetic Biology Came From and Where It Needs to Go*. Cell, 2014. **157**(1): p. 151-161.
14. Solomon, K.V. and K.L. Prather, *The zero - sum game of pathway optimization: Emerging paradigms for tuning gene expression*. Biotechnology journal, 2011. **6**(9): p. 1064-1070.
15. Farmer, W.R. and J.C. Liao, *Improving lycopene production in Escherichia coli by engineering metabolic control*. Nature biotechnology, 2000. **18**(5): p. 533-537.
16. Kobayashi, H., et al., *Programmable cells: interfacing natural and engineered gene networks*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(22): p. 8414-8419.
17. Tsao, C.-Y., D.N. Quan, and W.E. Bentley, *Development of the quorum sensing biotechnological toolbox*. Current Opinion in Chemical Engineering, 2012. **1**(4): p. 396-402.
18. Fuqua, W.C., S.C. Winans, and E.P. Greenberg, *Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators*. Journal of bacteriology, 1994. **176**(2): p. 269.
19. Surette, M.G. and B.L. Bassler, *Quorum sensing in Escherichia coli and Salmonella typhimurium*. Proceedings of the National Academy of Sciences, 1998. **95**(12): p. 7046-7050.
20. Bentley, W.E., et al., *Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria*. Biotechnology and bioengineering, 1990. **35**(7): p. 668-681.
21. DeLisa, M.P., J.J. Valdes, and W.E. Bentley, *Quorum signaling via AI-2 communicates the "Metabolic Burden" associated with heterologous protein production in Escherichia coli*. Biotechnology and bioengineering, 2001. **75**(4): p. 439-450.

22. Tsao, C.-Y., et al., *Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of E. coli*. Metabolic engineering, 2010. **12**(3): p. 291-297.
23. Tsao, C.-Y., et al., *LuxS Coexpression Enhances Yields of Recombinant Proteins in Escherichia coli in Part through Posttranscriptional Control of GroEL*. Applied and Environmental Microbiology, 2011. **77**(6): p. 2141-2152.
24. Kuipers, O.P., et al., *Quorum sensing-controlled gene expression in lactic acid bacteria*. Journal of Biotechnology, 1998. **64**(1): p. 15-21.
25. Tamsir, A., J.J. Tabor, and C.A. Voigt, *Robust multicellular computing using genetically encoded NOR gates and chemical 'wires'*. Nature, 2011. **469**(7329): p. 212-215.
26. Wood, T.K., S.H. Hong, and Q. Ma, *Engineering biofilm formation and dispersal*. Trends in Biotechnology, 2011. **29**(2): p. 87-94.
27. Basu, S., et al., *Spatiotemporal control of gene expression with pulse-generating networks*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(17): p. 6355-6360.
28. Quan, D.N. and W.E. Bentley, *Gene network homology in prokaryotes using a similarity search approach: Queries of quorum sensing signal transduction*. PLoS computational biology, 2012. **8**(8): p. e1002637.
29. Pereira, C.S., et al., *Identification of Functional LsrB-Like Autoinducer-2 Receptors*. Journal of bacteriology, 2009. **191**(22): p. 6975-6987.
30. Xavier, K.B. and B.L. Bassler, *Interference with AI-2-mediated bacterial cell-cell communication*. Nature, 2005. **437**(7059): p. 750-753.
31. Carter, K.K., J.J. Valdes, and W.E. Bentley, *Pathway engineering via quorum sensing and sRNA riboregulators—interconnected networks and controllers*. Metabolic engineering, 2012. **14**(3): p. 281-288.
32. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning*. Vol. 2. 1989: Cold spring harbor laboratory press New York.
33. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases*. Nat Meth, 2009. **6**(5): p. 343-345.
34. Wang, L., et al., *Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in Escherichia coli*. Journal of bacteriology, 2005. **187**(6): p. 2066-2076.
35. Bassler, B.L., E.P. Greenberg, and A.M. Stevens, *Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi*. Journal of bacteriology, 1997. **179**(12): p. 4043-4045.
36. Zargar, A., et al., *Bacterial Secretions of Nonpathogenic Escherichia coli Elicit Inflammatory Pathways: a Closer Investigation of Interkingdom Signaling*. mBio, 2015. **6**(2).
37. Smith, J.A.I., et al., *Biological screening of a diverse set of AI-2 analogues in Vibrio harveyi suggests that receptors which are involved in synergistic agonism of AI-2 and analogues are promiscuous*. Chemical Communications, 2009(45): p. 7033-7035.
38. Liu, Y., et al., *Biofabricating Multifunctional Soft Matter with Enzymes and Stimuli - Responsive Materials*. Advanced Functional Materials, 2012. **22**(14): p. 3004-3012.
39. Wu, H.C., et al., *Autonomous bacterial localization and gene expression based on nearby cell receptor density*. Molecular systems biology, 2013. **9**(1).
40. Hooshangi, S. and W.E. Bentley, *LsrR quorum sensing "switch" is revealed by a bottom-up approach*. PLoS computational biology, 2011. **7**(9): p. e1002172.
41. Taga, M.E., S.T. Miller, and B.L. Bassler, *Lsr-mediated transport and processing of AI-2 in Salmonella typhimurium*. Molecular Microbiology, 2003. **50**(4): p. 1411-1427.
42. Xue, T., et al., *LsrR-binding site recognition and regulatory characteristics in Escherichia coli AI-2 quorum sensing*. Cell Res, 2009. **19**(11): p. 1258-1268.

43. Xavier, K.B., et al., *Phosphorylation and Processing of the Quorum-Sensing Molecule Autoinducer-2 in Enteric Bacteria*. ACS Chemical Biology, 2007. **2**(2): p. 128-136.
44. Marques, J.C., et al., *Processing the Interspecies Quorum-sensing Signal Autoinducer-2 (AI-2): Characterization of phospho-(S)-4,5-dihydroxy-2,3-pentanedione isomerization by LsrG Protein* Journal of Biological Chemistry, 2011. **286**(20): p. 18331-18343.
45. Li, J., et al., *A stochastic model of Escherichia coli AI-2 quorum signal circuit reveals alternative synthesis pathways*. Molecular systems biology, 2006. **2**(1).
46. Studier, F.W., et al., *Understanding the Differences between Genome Sequences of Escherichia coli B Strains REL606 and BL21(DE3) and Comparison of the E. coli B and K-12 Genomes*. Journal of Molecular Biology, 2009. **394**(4): p. 653-680.
47. Barrios, A.F.G., et al., *Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motility quorum-sensing regulator (MqsR, B3022)*. Journal of bacteriology, 2006. **188**(1): p. 305-316.
48. Hegde, M., et al., *Chemotaxis to the quorum-sensing signal AI-2 requires the Tsr chemoreceptor and the periplasmic LsrB AI-2-binding protein*. Journal of bacteriology, 2011. **193**(3): p. 768-773.
49. Lu, T.K., A.S. Khalil, and J.J. Collins, *Next-generation synthetic gene networks*. Nat Biotech, 2009. **27**(12): p. 1139-1150.
50. Shong, J., M.R. Jimenez Diaz, and C.H. Collins, *Towards synthetic microbial consortia for bioprocessing*. Current Opinion in Biotechnology, 2012. **23**(5): p. 798-802.
51. Marchand, N. and C.H. Collins, *Peptide - based communication system enables Escherichia coli to Bacillus megaterium interspecies signaling*. Biotechnology and bioengineering, 2013. **110**(11): p. 3003-3012.
52. Roy, V., B.L. Adams, and W.E. Bentley, *Developing next generation antimicrobials by intercepting AI-2 mediated quorum sensing*. Enzyme and Microbial Technology, 2011. **49**(2): p. 113-123.
53. Hooshangi, S. and W.E. Bentley, *From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications*. Current Opinion in Biotechnology, 2008. **19**(6): p. 550-555.
54. Aurand, T.C., M.S. Russell, and J.C. March, *Synthetic signaling networks for therapeutic applications*. Current Opinion in Biotechnology, 2012. **23**(5): p. 773-779.
55. Roy, V., et al., *Cross species quorum quenching using a native AI-2 processing enzyme*. ACS Chemical Biology, 2010. **5**(2): p. 223-232.
56. Pereira, C.S., J.A. Thompson, and K.B. Xavier, *AI-2-mediated signalling in bacteria*. FEMS microbiology reviews, 2013. **37**(2): p. 156-181.
57. Xavier, K.B. and B.L. Bassler, *Regulation of Uptake and Processing of the Quorum-Sensing Autoinducer AI-2 in Escherichia coli*. Journal of bacteriology, 2005. **187**(1): p. 238-248.

Reaction	Differential Equation
Extracellular AI-2	$\frac{dAI2_{out}}{dt} = -k_{in} * (LsrACDB) * (AI2_{out}) + k_{out} * (AI2_{in})$
Intracellular AI-2	$\frac{dAI2_{in}}{dt} = k_{in} * (LsrACDB) * (AI2_{out}) - k_{out} * (AI2_{in}) - k_p * (LsrK) * (AI2_{in})$
LsrACDB protein synthesis	$\frac{dLsrACDB}{dt} = [K_{nat} + K_I * (IPTG_{ACDB})] * (OD_{600}^{ACDB}) - k_d * (LsrACDB)$
LsrK protein synthesis	$\frac{dLsrK}{dt} = [K_{nat} + K_I * (IPTG_K)] * (OD_{600}) - k_d * (LsrK)$
Cell density (LsrACDB overexpression)	$\frac{dOD_{600}^{ACDB}}{dt} = \mu_T * (OD_{600}^{ACDB})$
Cell density	$\frac{dOD_{600}}{dt} = \mu * (OD_{600})$

Table 1: Ordinary differential equations used to model the uptake of exogenously added AI-2 by each plasmid in the strain LW12

<i>Species</i>	<i>Description</i>	<i>Initial Condition or Range</i>
t	Time	$[0, 270] \text{ min}$
$AI2_{out}$	Extracellular AI-2	$50 \mu M$
$AI2_{in}$	Intracellular AI-2	$0 \mu M$
$IPTG_K$	Plasmid-encoded LsrK	$[1, 0]$
$IPTG_{ACDB}$	Plasmid-encoded LsrACDB	$[1, 0]$
$LsrK$	Lsr Kinase	$0 \mu M$
$LsrACDB$	Lsr ACDB transporter	$0 \mu M$
OD_{600}^{ACDB}	Cell density (pLsrACDB, pLsrACDBFG)	$0.4-0.6$
OD_{600}	Cell density (pLsrK, pLsrFG, empty)	$0.4-0.6$
<i>Parameters</i>	<i>Description</i>	<i>Best fit value</i>
k_{in}	AI-2 import by LsrACDB complex	$0.008 \mu M^{-1} \text{ min}^{-1}$
k_{out}	AI-2 export	0.045 min^{-1}
k_p	AI-2 phosphorylation	$0.006 \mu M^{-1} \text{ min}^{-1}$
K_I	Induced expression	$0.9 \mu M^{-1} \text{ min}^{-1}$
K_{nat}	Native expression	$0.1 \mu M^{-1} \text{ min}^{-1}$
k_d	Protein decay	0.02 min^{-1}
μ	Growth rate	0.0056 min^{-1}
μ_T	Growth rate (LsrACDB expression)	0.0044 min^{-1}

Table 2: Model species and kinetic rate constants in model of exogenously added AI-2 uptake by each plasmid in the strain LW12

Scheme 1: Panel (A) depicts the AI-2 quorum sensing network. *LuxS* generates AI-2 from metabolic precursors, which is then exported out of the cell by *TqsA*. AI-2 is primarily taken up through the ABC-type transporter *Lsr*, and then phosphorylated by *LsrK* to AI-2P. AI-2P depresses the response regulator *LsrR*, thereby activating transcription of the *lsr* operon. AI-2P is degraded by *LsrF* and *LsrG*. Panel (B) depicts the ‘controller cells’ that are engineered through the overexpression of distinct components of the *lsr* system. “Native” indicates native production, while ‘Induced’ indicates over-expression. Panel (C) illustrates the strains, plasmids, descriptions and sources used for these ‘controller cells’.

Figure 1: A) AI-2 uptake profiles of 'controller cells'. Each plasmid was transformed into LW12 (W3110 *AluxS*) and grown to $OD \sim 0.4$ before the addition of IPTG (1 mM) and AI-2 (50 μM). AI-2 levels were measured with AI-2 activity assays (see Methods) every 30 minutes (data points) and a mathematical model was created (black trendlines) to fit the data. B) Collation of mathematical models of each strain. Each experiment is performed in triplicate.

Figure 2: Cell-cell modulation of protein expression. Panel (A) shows results for two cultures with an initial state of non-fluorescence. A 1:1 mixture of OD~1 of LW12 pLsrACDBFG or LW12 pTrcHisB is mixed with OD~0.2 of reporter strain W3110 pCT6 pET-GFP. Panel (B) shows FACS data of the non-fluorescing population of both mixtures and the results 10 hours later. Microscopic images of the cells at 10 hours are shown, adjusted for clarity.

A

B

Figure 3: LW12 pLsrACDBFG modulates AI-2 in the microenvironment. Panel (A) illustrates the AI-2 in co-cultures of *E. coli* BL21 (*luxS*⁺) with LW12 pTrcHisB (blue) and LW12 pACDBFG (red), respectively, over time in a range of concentration ratios. AI-2 levels were measured with AI-2 activity assays every 30 minutes. Each experiment is performed in triplicate with error bars indicating standard error. Panel (B) compares the AI-2 activities at 120 minutes with the mathematical model generated.

Figure 4: Effects of AI-2 on biofilm production. Co-incubations of (A) W3110 pTrcHisB : LW12 pTrcHisB (B) W3110 pTrcHisB : LW12 pTrcHisB + homocysteine (50 μ M) (C) W3110 pTrcHisB : LW12 pTrcHisB + AI-2 (50 μ M) (D) W3110 pTrcHisB : LW12 pLsrACDBFG (E) W3110 pTrcHisB : LW12 pLsrACDBFG + homocysteine (50 μ M) (F) W3110 pTrcHisB : LW12 pLsrACDBFG + AI-2 (50 μ M) are mixed at a 1:1 ratio at OD ~ 0.05 and IPTG (1 mM) is added at OD ~ 0.4. Homocysteine is added as a negative control to evaluate the effect of exogenous AI-2. Biomass is measured in technical triplicate after 24 hours, and each experiment is performed in biological duplicate. Figure 4A illustrates optical density at OD₆₀₀ after 24 hours. Figure 4B shows the ratio of cell density at OD₆₀₀ to biomass measured at OD₅₄₀. A Student's two tailed unpaired t-test was used to compare the groupings shown and a significance value of $p = 4.85 \times 10^{-5}$ was determined. All error bars indicate one standard deviation.

Figure 5: Effects of AI-2 on chemotaxis. Migration of CT104 (*AluxS Δ lrfG*) in a transwell incubated for two hours (see Methods) with induced (1 mM IPTG) conditioned media of (A) LW12 *pTrcHisB* (B) LW12 *pTrcHisB* + AI-2 (20 μ M) (C) LW12 *pLsrACDBFG* and (D) LW12 *pLsrACDBFG* + AI-2 (20 μ M). Each experiment is performed in biological triplicate. Error bars indicate one standard deviation.