Can the natural diversity of quorum-sensing advance synthetic biology?

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Quorum-sensing networks enable bacteria to sense and respond to chemical signals produced by neighboring bacteria. They are widespread: over 100 morphologically and genetically distinct species of eubacteria are known to use quorum sensing to control gene expression. This diversity suggests the potential to use natural protein variants to engineer parallel, input-specific, cell–cell communication pathways. However, only three distinct signaling pathways, Lux, Las, and Rhl, have been adapted for and broadly used in engineered systems. The paucity of unique quorum-sensing systems and their propensity for crosstalk limits the usefulness of our current quorum-sensing toolkit. This review discusses the need for more signaling pathways, roadblocks to using multiple pathways in parallel, and strategies for expanding the quorum-sensing toolbox for synthetic biology.

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The simplicity of these networks allows researchers to model how quorum-sensing-controlled gene expression is regulated in response to HSL signal concentration (McMillen et al., 2002; Pai and You, 2009; Pai et al., 2014). These models can inform how a quorum-sensing network should be implemented in a synthetic circuit to achieve the desired behavior (McMillen et al., 2002; Pai and You, 2009). Furthermore, dry lab researchers have used modeling to demonstrate how quorum-sensing systems control group response in the presence of noisy signal concentrations, supporting their use in synthetic biology as robust circuit components (Koseska et al., 2009; Weber and Buceta, 2013).

Incorporating quorum-sensing networks into production strains has advanced the field of metabolic engineering. Quorum sensing has been used to synchronize gene expression across a population to reduce cell-to-cell variability and to increase yields in engineered strains (Danino et al., 2010; Prindle et al., 2012a,b; Anesiadis et al., 2013). For example, by linking a Lux-based genetic oscillator with a gas phase signal oscillator, researchers coordinated gene expression among 2.5 million cells across 5 mm of space with minimal noise (Danino et al., 2010; Prindle et al., 2012a,b), Anesiadis et al. (2013) employed this type of circuit in a production strain, where they engineered a cell-density-dependent switch using the Lux system to control production of serine in an *Escherichia coli* knockout strain. Group-controlled gene expression implemented by an HSL quorum-sensing network leads to overall higher serine production.

Quorum-sensing networks are also used in genetic circuits to perform computation. Tabor et al. (2009) took advantage of the diffusibility of HSL through agar to build a bacterial edge detector using the Lux network. They demonstrated that stationary physical spacing of bacteria relative to different inputs drives controlled expression of an output. The circuit was designed such that bacteria exposed to darkness expressed HSLs but no output (LacZ). The circuit allowed only bacteria that were both adjacent to HSL-producers and exposed to light to express LacZ, which resulted in a pigmented outline at the edges of a light-masked region (Figure 2B). While most bio computation is digital, Daniel et al. (2013) showed the versatility of quorum-sensing networks by demonstrating analog computation using the Lux network: their circuit converts logarithmic HSL input into linear fluorescent output over a large range of HSL concentrations. Thus far, engineered...
bio computation has used monolayers of cells. Three-dimensional (3D) colony-printing techniques will increase the sophistication of these systems (Connell et al., 2013). Controlled spacing of colonies based on HSL-diffusion rates could allow engineering a temporal element into a split circuit.

In the preceding examples, the cells in each system are expressing the same circuit. However, engineers may also coordinate gene circuits distributed among multiple populations. Brenner et al. (2007) used the Rhl and Las networks from *Pseudomonas aeruginosa* to build two strains of *E. coli* that form a biofilm together once both populations reach a threshold density. Balagaddé et al. (2008) used components from the Lux and Las networks to engineer a predator–prey relationship between *E. coli* strains. High predator population density induces cell death in the prey strain, while high prey population density supports survival of the predator strain. You et al. (2004) placed a cell death gene under the control of the Lux promoter and built a bistable system that maintains a population density of a defined range. At high cell density, the Lux network activates a cell death. At decreased cell density, the cell death gene is inactive and the population begins to grow again. Computation may be split across multiple strains, distributing the energy demands of a complex computation that are too great for a single cell (Ji et al., 2013; Payne and You, 2014) (Figure 2A). Wang et al. (2013) built a two-strain, three-input biosensor in *E. coli* that produces RFP only in the presence of three heavy metal contaminants: arsenic, mercury, and copper. Cell 1 produces LuxI after exposure to arsenic and mercury; Cell 2 expresses RFP in response to 3O-C6-HSL produced by Cell 1 and copper. Tamsir et al. (2011) linked circuits expressed in multiple cell populations using two quorum-sensing networks derived from *P. aeruginosa*, Rhl and Las (Figure 2C). They implemented complex Boolean expressions by linking combinations of small molecule inputs (e.g., tetracycline aTc and arabinose Ara) to outputs (e.g., yellow fluorescence) using HSL signaling (Tamsir et al., 2011).

**CROSSTALK BETWEEN QUORUM-SENSING PATHWAYS CHALLENGES THE DEVELOPMENT OF SYNTHETIC GENETIC CIRCUITS**

Attempts to isolate, study, and apply quorum-sensing pathways for bioengineering is often thwarted by unexpected crosstalk. Quorum sensing is a popular tool among synthetic biologists for designing multicellular systems, but widely utilized HSL quorum-sensing networks are currently limited to three pathways: Lux, Las, and Rhl. These networks all exhibit crosstalk with each other, complicating the design of complex genetic systems implemented with quorum-sensing networks.

For instance, a single regulator can be activated by multiple acyl-HSL-class molecules, resulting in cross-activation of regulators from different species of bacteria. This phenomenon was observed in a proof-of-concept system designed by Canton et al. (2008) wherein an output gene for green fluorescent protein (GFP) was placed under the control of a LuxR receiver module (Wu et al., 2014). Four chemically distinct acyl-HSLs, C6-HSL, C7-HSL, 3O-C8-HSL and, at higher concentrations, C8-HSL, all activated expression of GFP at levels comparable to or even greater than the cognate LuxI acyl-HSL, 3O-C6-HSL (Canton et al., 2008). Many
different HSL synthases, including EsaI, Expl, and AhII, produce the same major cognate acyl-HSL as LuxI, suggesting that these pathways would have high levels of crosstalk if built into the same network (Miller and Bassler, 2001; Põllumaa et al., 2012). In the report of their predator–prey, two-strain system, Balagadde et al. (2008) discussed low-level crosstalk between LuxI and LasR, which was recently confirmed by observing Lux and LasR interactions in a single-strain system (Wu et al., 2014). However, Balagadde’s system functioned such that crosstalk was apparently below the threshold for altering intended behavior. Interestingly, this type of crosstalk is also observed in nature (Fuqua et al., 1996). For example, two opportunistic pathogens, Burkholderia cepacia and P. aeruginosa, are known to co-infect patients with cystic fibrosis (Lewenza et al., 2002). Each pathogen’s quorum-sensing regulators respond to the other’s HSLs, resulting in coordination of virulence-gene expression.

Crosstalk can also occur at the level of the target, or “output,” gene; similarities between promoter sequences and the DNA-binding domains within the regulator proteins contribute to crosstalk between quorum-sensing pathways. The acyl-HSL-activated LuxR regulator stimulates transcription at its cognate promoter as well as the Esa promoter, while acyl-HSL-activated LasR, EsaR, and ExpR regulators are also capable of initiating transcription at the Lux promoter (von Bodman et al., 2003; Saedidi et al., 2011; Shong et al., 2013). While this type of crosstalk can be avoided by using only one regulator per strain, they will not behave as two orthogonal wires within a single cell.

EXPANDING THE SET OF ORTHOGONAL QUORUM-SENSING PATHWAYS ENABLES DESIGN OF COMPLEX GENETIC CIRCUITS

Synthetic circuits may be engineered to detect specific combinations of input signals so long as each sensing pathway functions independently (orthogonally) without undesired intercommunication (crosstalk). Genetic circuits designed to respond to complex combinations of environmental conditions must distinguish and integrate multiple distinct input signals. Orthogonal quorum-sensing pathways are necessary to implement complex circuits that respond to signals produced by living cells, rather than requiring synthetic, exogenous inputs. Engineered division of labor is a major research area in metabolic engineering (Bernstein et al., 2012; Vinuselvi and Lee, 2012); orthogonal quorum-sensing modules will enable further development of cell-autonomous metabolic regulation in multi-strain bioreactor systems. Quorum-sensing circuits could be used to engineer multi-strain, self-monitoring microbial populations that perform energetically expensive metabolic processes in a single culture. Multiple co-cultured strains could be designed to monitor and maintain a target population ratio, or steps in a metabolic process could be timed for accumulation of precursors (Tamsir et al., 2011).

Circuit sophistication is limited by metabolic capacity, transcription and translation resources, and crosstalk within the cell. Moon et al. (2012) pushed the bounds of single-cell computational capability by building a four input AND gate in E. coli. Their complex logic gate allows living bacterial cells to express GFP in the presence of four exogenous compounds and no fewer. Transcription activator complexes were decoupled and placed under the control of distinct inducible promoters that respond to the presence of soluble compounds (arabinose, IPTG, tetracycline, and the acyl-HSL 3-O-C6-homoserine lactone) in the cell culture medium. More complex circuits could be implemented by replacing the exogenous inputs in the Moon et al. system with quorum-sensing wires linking cells performing independent computation. Scaling can be achieved through modularity by building complex computational systems with simple independent components. By designing the components in separate strains and connecting them with orthogonal quorum-sensing wires, computational steps can be performed independently without exhausting cellular resources.

Connecting complex circuits requires orthogonal HSL networks to independently signal each strain’s computation. However, using even two quorum-sensing networks in parallel is constrained by crosstalk. To our knowledge, there is no published demonstration of three or more orthogonal quorum-sensing networks in a single system. The complexity of multi-input integration circuits remains constrained by reliance on exogenous signals and by the limited number of orthogonal input–output pathways.

STRATEGIES FOR MINIMIZING CROSSTALK

Promiscuous interactions between HSLs and regulators, as well as between regulators and promoters, prevent many quorum-sensing systems from operating independently and in parallel. Some have used gene–network engineering approaches to mitigate crosstalk (Brenner et al., 2007; Balagadde et al., 2008; Tamsir et al., 2011; Wu et al., 2014). For instance, Brenner et al. (2007) engineered their system to avoid crosstalk between the Las and Rhl networks. They split the networks between two strains to eliminate promoter–regulator crosstalk and controlled HSL syntheses production via a positive feedback loop to achieve a two-strain, biofilm-forming consortium.

Another approach to eliminate crosstalk between signaling pathways is using quorum-sensing pathways from distinct families (the aforementioned HSL, AI-2, and AIP pathways). Significant variance in the chemistry of the signaling molecules suggests that cross-reactivity is unlikely: HSLs contain a lactone ring with a hydrocarbon acyl or aeryl tail, AI-2 is a furanosyl borate diester composed of two five-membered rings stabilized by a boron atom, and AIPs are relatively large circular peptides composed of amino acids (Chen et al., 2002; Marchand and Collins, 2013). However, this approach may be limited in its flexibility since both AI-2 and AIP require active transport and multiple proteins to generate and detect the signals. With a few exceptions, HSL networks require only two proteins and one promoter. While AI-2 quorum sensing is limited to only one signaling molecule, multiple AIP pathways may exist that do not have cross-reactivity. Marchand and Collins (2013) recently demonstrated modularity and orthogonality of two AIP signals from Staphylococcus aureus. In their system, E. coli was the AIP sender, producing and secreting two AIPs, and two engineered strains of Bacillus megaterium each received one of the signals but not the other. While the ability to use two AIPs in a single cell was not explored, this is a promising result and further research could demonstrate orthogonality between AIPs and HSL quorum sensing.
Directed evolution could also be used to generate regulator proteins that specifically respond to any desired HSL. Mutational analyses and 3D protein structure data have helped to identify key amino acid residues that govern the interaction between regulators and acyl-HSL ligands. Using positive and negative selection, Collins et al. (2006) generated a LuxR mutant that no longer responds to the cognate 3O-C6-HSL but gained responsiveness to C10-HSL, to which wild-type LuxR does not respond. They then demonstrated the orthogonality of LuxR wild type versus the mutant. However, directed evolution of regulator proteins to generate novel orthogonality is technically daunting and only generates mutants with minor changes to the wild-type binding pocket, limiting the range of possible novel behaviors. Furthermore, they bind to and activate the same promoter and, while this feature could be leveraged to build OR gates, they cannot be used as orthogonal networks in the same cell without further mutagenesis to alter promoter-binding specificity.

Finally, scientists could explore other microbial genomes for quorum-sensing homologs that have not yet been exploited for synthetic biology. Comparative genomics has identified dozens of HSL family (Lux-like) homologs in divergent species (Case et al., 2008). A major advantage of exploring wild-type homologs over directed evolution is that natural evolution has already “discovered” functional regulators in a very broad exploration space of amino acid sequences. Evolution has selected for regulator proteins of significantly different sizes, as opposed to artificial selection techniques that, due to practical constraints, do not deviate significantly from pre-existing primary structures.

**THE BASIS OF SPECIFICITY IN THE HSL SIGNALING FAMILY**

Investigations of microbial quorum-sensing pathways have revealed molecular characteristics that underlie the diversity of HSL signaling pathways in different species. These signaling pathways have been distinguished on the basis of the operator binding regions in different regulators, we have generated scaled protein domain maps using descriptions from the literature (Egland and Greenberg, 2001; Zhang et al., 2002; Bottomley et al., 2007; Chen et al., 2011) and annotations from protein domain-scanning databases Uniprot (UniProt Consortium, 2014), Prosite (Sigrist et al., 2013), InterPro (Mitchell et al., 2015), and the Protein Data Bank (Berman et al., 2000) (Figure 5). Autoinducer-binding regions (InterPro IPR005143) contain roughly six alpha helices and six beta strands. Published 3D structures for TraR (PDB 1L3L) (Zhang et al., 2002), LasR (PDB et al., 2UV0) (Bottomley et al., 2007), CviR (PDB 3QP6) (Chen et al., 2011), and SdiA (Yao et al., 2006) reveal that a five-strand beta sheet is sandwiched...
between two three-helix bundles. The C-terminal DNA-binding domains are characterized as “helix–turn–helix” (HTH) regions (Prosite PS00043) that consist of four alpha helices. The second and third helices within the HTH region are often identified as a conserved H–T–H motif (Prosite PRU00411); the third helix has been characterized as the DNA recognition helix in TraR (Zhang et al., 2002). When HSL molecules bind to their corresponding quorum-sensing regulator, they often induce multimerization of regulator proteins. This multimeric state is the active form, capable of binding an inverted DNA sequence repeat at the target promoter and inducing transcription of downstream genes.

Analysis of the HSL–regulator binding pockets suggests that the shape, size, hydrophobicity, and functionalization determine the binding affinity of a regulator for a specific HSL. This implies that comparison of known HSL–regulator interactions may identify likely candidates for orthogonal quorum-sensing networks. For example, it has been hypothesized that quorum-sensing systems that produce long, straight-chain acyl-HSLs have regulators with longer binding pockets; likewise, a system that uses acyl-HSL molecules with branching functional groups will have regulators with binding pockets that accommodate the branches (Bottomley et al., 2007). Thus, taking steric into account, a quorum-sensing system that uses HSL molecules with a relatively short hydrocarbon tail and bulky functional groups may be orthogonal to a system that uses long-chain, non-branched HSL molecules.

Hydrophobic interactions between the HSL tail and amino acid residues within the binding pocket suggest that these binding interactions are dominated by van der Waals forces (Bottomley et al., 2007) (Figure 3). Because the HSL tail is buried within the binding
FIGURE 5 | Comparison of protein motifs in select regulators. The three-dimensional (3D) structure of TraR is shown as an example of how domains and the homoserine lactone (HSL) ligand are typically positioned in space. The underlined letters in the b–b–a–a–b–a–b–b secondary structure motif indicate the location of highly conserved amino acids that form hydrogen bonds with the homoserine lactone head of HSLs. Published 3D structure data (Protein Data Bank) are listed where available (“–” = not available). Abbreviations used are: Reg. = regulator protein, H–T –H = helix–turn–helix, a = alpha helix, b = beta strand, h = 3/10 helix.

Database entries for conserved motifs are: autoinducer binding = IPR005143, HTH LuxR-type = PS50043. Inferred binding pockets are patterns of secondary structures that are similar to the TraR binding pocket. Inferred recognition helices are the second alpha helix from the C-terminus. Secondary structures for proteins with no available 3D structure data were mapped using the Jpred prediction tool (Cole et al., 2008). Maps were generated using DomainDraw (Fink and Hamilton, 2007).

Pharmacophore models for HSL-regulator binding developed by Geske et al. (2008) support the idea that functionalization of the HSL molecule underlies binding pocket selectivity. Their models are based on the response of Tra, Las, and Lux regulators to libraries of HSLs and synthetic analogs in a system that used beta-galactosidase as the output gene. Comparison of the atomic geometries of ligands reveals three general properties linked with HSL efficacy: spacing of hydrophobic regions, hydrogen bond donor regions, and hydrogen bond acceptor regions within the R-group attached to the lactone ring. For instance, TraR shows the greatest response to a group of ligands in which the acyl tail contains one hydrogen bond donor region followed by two hydrogen bond acceptor regions arranged in trans and ended in a hydrophobic region (Geske et al., 2008).

Conservation and divergence in the conformation of regulator N-terminal HSL-binding regions support the idea that variation in HSL R-groups coordinates selective regulator–ligand interactions. Here, we explore whether motifs in the protein structures of regulators provide insight into the underlying mechanism of HSL-binding selectivity. Primary sequence alignments show 10–25% identity in regulator homologs (Bottomley et al., 2007) and therefore provide very limited information. We have attempted a more coarse-grained approach on a select set of well-characterized regulators by annotating secondary structures that correspond to the TraR binding pocket. For regulators that lack published 3D structure data, we have annotated secondary structures as hypothetical HSL-binding pockets (Figure 5).
The autoinducer-binding region contains two functional domains in its tertiary structure: the multimerization surface and the HSL-binding pocket. The multimerization surface of the TraR homodimer consists primarily of alpha helices a1 and a6 (Bottomley et al., 2007), plus other residues within loops that link helices and beta strands (Figure 5). The HSL-binding pocket binds a single HSL molecule in the space between a five-strand antiparallel beta sheet and a three-helix bundle (Bottomley et al., 2007).

In the primary structure of TraR, these secondary structures are arranged in the order of b–b–a–a–b–a–b–b. The first and second alpha helix and the last beta strand of this motif (underlined in Figure 5) contain the amino acids that form hydrogen bonds with the homoserine lactone head of the HSL ligand. These residues are highly conserved in LuxR protein homologs, reflecting a common binding mechanism at the non-variable head regions of HSL molecules. In contrast, the variable acyl tail extends into the region of the binding pocket that is formed by residues that show less conservation in LuxR homologs, suggesting a mechanism for HSL selectivity (Bottomley et al., 2007).

TraR and SdiA are most responsive to the ligand 3O-C8-HSL (Michael et al., 2001; Geske et al., 2008). These regulators contain the same b–b–a–a–b–b–b pattern of secondary structures in their HSL-binding pocket domains (Figure 5). This pair of regulators fits the attractive idea that binding pockets with similar secondary structures may prefer the same HSL ligands. However, comparisons of other regulators challenge this idea. While some regulators that respond to HSLs with smaller or larger R-groups deviate from the b–b–a–a–b–b–b motif, there are others, i.e., RhIR, LasR, and SinR, which contain the same motif yet respond to different ligands: C4-HSL, 3O-C12-HSL, and C18-HSL, respectively (Llamas et al., 2004; Kumari et al., 2006; Geske et al., 2008). It is possible that variations in specific residues in RhIR, LasR, and SinR account for their preferences for different ligands. AubR contains a substitution of the third beta strand with an alpha helix in the b–b–a–a–b–b–b motif, similar to LuxR and BjaR. LuxR and BjaR respond to 3O-C6-HSL (Canton et al., 2008) and isovaleryl-HSL (Lindemann et al., 2011), respectively. Assuming that the ligand for AubR is C12-HSL [produced by AubI (Nasuno et al., 2012)], AubR, LuxR, and BjaR represent another set of regulators where similarities in secondary structures do not appear to correspond to similar ligands. Exploration of the range of HSL responsiveness of these regulators may provide more insight into their structure–function relationships.

Interestingly, no HSL-regulator protein-related motifs appear in GtaR. Leung et al. (2012) reported that GtaR regulates its target promoter (a Lux promoter homolog) in response to C16-HSL and cell-free growth medium collected from HSL-producing strains. GtaR shows sequence conservation with the TatD family of deoxyribonuclease proteins. Like the LuxR homologs, TatD proteins contain interspersed beta strands and alpha helices. Here, we have annotated predicted secondary structures within GtaR; these domains are inferred from comparisons of GtaR with closely related TatD proteins that have published 3D structures. Given its distinct arrangement of secondary structures, GtaR might represent a unique class of HSL-responsive regulator proteins.

CONCLUSION AND DISCUSSION

The information we present here on the diversity of HSL molecules and regulator proteins is insufficient to conclude that the structures of regulator binding pockets and the atomic geometries of the HSL ligands imply orthogonality. Regulators that respond to distinct HSL ligands show different protein folding patterns in some cases but similar structures in others. With limited data on regulator promiscuity, secondary structure alone cannot predict HSL ligand preference; thus, interaction between specific amino acid residues and atoms within the HSL molecule may need to be considered. This investigation is limited by the lack of 3D structure data for LuxR homologs.

Many gaps in knowledge remain in understanding the extent of orthogonality or interaction between the homologous pathways in living cells. To date, the published functional studies of the quorum-sensing homologs in synthetic circuits (HSL synthases, regulators, and promoters) include just three homologous quorum-sensing pathways, or they use purified compounds to stimulate one or a few regulators. Furthermore, the available 3D structure data for regulator proteins is sparse compared to the total number of putative regulator homologs that have been identified via metagenomic analysis (Nasuno et al., 2012). More comprehensive analyses to study the responses of regulator proteins to different HSLs, such as those of Geske et al. (2008), may enable us to predict and select orthogonal pathways for use in complex synthetic systems. For instance, E. coli could be used as a universal host to carry dozens of decoupled sender and receiver components (Figure 1), derived from the genomes of various bacterial species. Culture media from sender strains could be used to stimulate receiver strains carrying a reporter driven by a receiver system (regulator protein and its corresponding promoter).

The discovery of novel orthogonal quorum-sensing pathways will provide metabolic engineers and synthetic biologists with HSL signaling wires that do not cross-react. Using these insulated, independently functioning pathways, synthetic circuits could be designed to detect distinct combinations of multiple input signals and scale simple single-cell components to sophisticated multi-strain circuits. It is imperative to continue research on quorum-sensing pathway behavior across multiple disciplines, including crystallography, molecular biology, microbiology, metabolic engineering, and synthetic biology, to fill critical gaps in knowledge that have prevented us from engineering highly sophisticated biological systems.

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