DNA6: Sixth International Meeting on DNA Based Computers

Engineered Communications for Microbial Robotics

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Abstract. Multicellular organisms create complex patterned structures from identical, unreliable components. Learning how to engineer such robust behavior is important to both an improved understanding of computer science and to a better understanding of the natural developmental process. Earlier work by our colleagues and ourselves on amorphous computing demonstrates in simulation how one might build complex patterned behavior in this way. This work reports on our first efforts to engineer microbial cells to exhibit this kind of multicellular pattern directed behavior.

We describe a specific natural system, the Lux operon of *Vibrio fischeri*, which exhibits density dependent behavior using a well characterized set of genetic components. We have isolated, sequenced, and used these components to engineer intercellular communication mechanisms between living bacterial cells.

In combination with digitally controlled intracellular genetic circuits, we believe this work allows us to begin the more difficult process of using these communication mechanisms to perform directed engineering of multicellular structures, using techniques such as chemical diffusion dependent behavior. These same techniques form an essential part of our toolkit for engineering with life, and are widely applicable in the field of microbial robotics, with potential applications in medicine, environmental monitoring and control, engineered crop cultivation, and molecular scale fabrication.

1. Introduction

The developmental process requires coordinated, robust action among a very large number of essentially identical, unreliable components. In stark contrast to current computer science engineering practice, these developmental programs are highly fault tolerant. Imagine what would happen if any biological mechanism exhibited the same fragility as a modern microprocessor, operating system, or satellite.

Previous work in our group [**1, 3, 4, 24, 26, 30, 29**] has looked at some of these robustness and pattern formation issues in simulation, with intriguing results. We found that the topic we call *amorphous computing* requires a different set of algorithms and a different approach to thinking about structures than conventional computer science.

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But we also must better understand the developmental process in a biological context. Although we are making significant progress, we simply do not fully understand the pattern formation of even the simplest of biological structures. But surely concepts from computer science, such as subroutines, divide-and-conquer, recursion and iteration will play a major role in understanding the genetic control of developmental diversity. Both biology and computer science have lessons to learn from a cooperative investigation of this field.

Even simple biological systems can exhibit complex developmental processes. The motile, gram negative bacterium *Myxococcus xanthus*, for example, exhibits social behavior and cellular differentiation during cooperative feeding. The controlled, density dependent, release of antibiotics and cell wall degrading enzymes to kill competitors allows moving swarms (so-called "wolf-packs") to act more effectively than individuals [**8**]. Similarly, *M. xanthus* exhibits selection, during starvation, of a small number of cells out of a swarm of 100,000 to change form from rod-like bacteria to environmentally protected spherical myxospores. Spore formation requires high cell density, nutrient limitation, and a solid surface [**9, 20**].

In this paper we undertake a *biological implementation* of what we believe is a key component in building such developmental pattern engineering techniques – cell to cell communications. Communication between cells is obviously essential to any kind of coordinated expression. But in development, and in our amorphous computing simulations, one kind of communication emerges as especially important – the ability to detect and act on chemical signal concentration gradients. Such gradient dependent expression is the building block of locally unique behavior, as well as the organizing principle which allows the construction of local coordinate systems through the creation and detection of chemical gradients. Such trophic behavior provides one basic organizing principle for complex patterned development.

In this work, we have isolated a specific chemical cell to cell signaling mechanism from a natural biological system, the quorum sensing system of *Vibrio fischeri*. This system encodes genes and promoter sequences which allow the controlled expression of the chemical *Vibrio fischeri* autoinducer (VAI) within one sender cell, and the detection and controlled expression of specific genes in another, receiving cell. The free diffusion of the VAI chemical within the medium and across cell membranes allows the establishment of chemical gradients and the controlled expression of genetic circuits as a result.

Specifically, we demonstrate in this work the construction and testing of engineered genetic circuits which exhibit the ability to send a controlled signal from one cell, diffuse that signal through the intercellular medium, receive that signal within an a second cell, and activate a remote transcriptional response.

In combination with other ongoing work in digitally controlled gene expression [**23, 19, 16, 11**] this work provides components for a biological substrate for expressing pattern formation. These same components are also a key part of our toolkit for engineering with life, with important implications for medicine, agriculture, environmental monitoring, and engineering – including molecular scale manufacturing and molecular electronics.

In the remainder of this paper we describe the mechanism of quorum sensing in bacteria (Sections 2-3), present the plasmids engineered for communications (Section 4), report on our experimental results (Section 5), and offer conclusions and avenues for future work (Section 6).

2. Quorum Sensing in bacteria

Vibrio fischeri is a gram-negative bioluminescent marine prokaryote which naturally occurs in two distinct environments. In seawater, it swims freely at concentrations of approximately ten cells per liter. It also grows naturally in a symbiotic relationship with a variety of invertebrate and vertebrate sea organisms, especially the Hawaiian sepiolid squid, *Euprymna scolopes* and the Japanese pinecone fish, *Monocentris japonica* [**27**]. In these symbiotic relationships, the bacteria grow to densities of approximately 10^{10} cells per liter.

In the free living state, *Vibrio fischeri* emits essentially no light (< 0.8 photons/second/cell). In the light organ, however, the same bacteria emit more than 800 photons/second/cell, producing very visible bioluminescence. In culture, *Vibrio fischeri* demonstrates a similar density dependent bioluminescence, with induction occurring at about 10^{10} cells/liter.

Work over many years has established that this behavioral change is due to a natural cell density detection mechanism, which has been termed quorum sensing [**15**]. The quorum sensing mechanism relies on the synthesis and detection of a very specific, species unique chemical, an *autoinducer*, which mediates intercellular communications. In *Vibrio fischeri*, this autoinducer chemical (VAI) has been identified as N-(3-oxohexanoyl)-3-amino-dihydro-2-(3H)-furanone [**10**]. The gene, LuxI, catalytic protein, and synthetic pathway for this chemical has also been identified [**14**].

Briefly, the LuxI gene encodes an acyl-homoserine lactone synthesase which uses highly available metabolic precursors found within most gram negative prokaryotic bacteria – acyl-ACP from the fatty acid metabolic cycle, and S-adenosylmethionine (SAM) from the methionine pathway – to synthesize VAI.

The *Vibrio fischeri* autoinducer (VAI) freely diffuses across the bacterial cell membrane. Thus, at low cell densities, low VAI concentrations are available. Within a light organ, or at high culture densities, VAI builds up within the environment, resulting in a density dependent induction of bioluminescence.

The response mechanism to VAI concentration has also been extensively analyzed [**28**]. Briefly, the LuxR gene codes for a two domain DNA binding protein which interacts with VAI and the Lux box of the LuxICDABEG operon promoter to exercise transcriptional control. At nanomolar concentrations, VAI binds to the N terminal domain of the LuxR protein, which in turn activates the C-terminal helix-turn-helix DNA binding domain. The LuxR protein acts as a transcriptional activator for the RNA polymerase holoenzyme complex. The activated protein likely binds in dimeric or multimeric forms, because of the evident dyadic symmetry of the Lux box binding domain.

The genetic structure of the *Vibrio fischeri* Lux operon has been established by the successful cloning and expression of the Lux genes into *E. coli* [**12**]. It is somewhat surprising (although common) for the transfer of regulatory genes and entire metabolic pathways to function straightforwardly across gram-negative species boundaries in this way.

Given the potential utility of both the autoinducer control mechanism as a cell to cell signaling mechanism, and the Lux operon as a reporter gene, we undertook to isolate these operons and engineer their interfaces. An initial stumbling block was the lack of complete sequence information. Remarkably, although this system has been the subject of hundreds of papers, a complete sequence of the operons

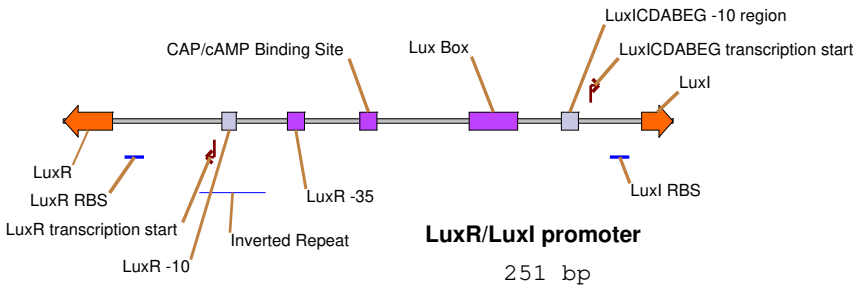

FIGURE 1. LuxR and LuxI promoter regions from *Vibrio fischeri*.

was not available in GENBANK. Therefore, as first step, we undertook to isolate the operon, completely sequence it, and deposit the resulting sequence. That effort is described in Appendix A.

3. Genetic Features of the LuxR/LuxI operons

The nucleotide structure of the sequenced regulatory region is shown in figure 1. This region encodes two divergently transcribed promoters. The left operon constitutively expresses the LuxR transcript, which is coded by the left ORF. This operon has a standard σ^{70} binding region, consisting of a -10 and -35 sequence, and a CRP/CAMP binding site. The CRP/CAMP binding site allows catabolic repression on the left LuxR operon.

The right operon drives expression of the LuxICDABEG transcript, coding for autoinducer production (LuxI) and the bioluminescence cassette of LuxCDABEG. It consists of a standard -10 σ^{70} binding site, but is missing the -35 site. Instead, the *lux box*, a 20 base inverted palidromic repeat, allows dimeric binding of the active form of LuxR binding protein, activating the RNA polymerase holoenzyme complex, under control of the LuxR protein – and hence indirectly, the VAI concentration.

The lux box is a common motif in regulatory proteins of the LuxR family, and occurs upstream of many LuxR homologous genes. The sequence of the Lux box in this construct is 5'(acctgtagga tcgtacaggt); the consensus sequence for similar lux boxes in other constructs is [**18**] 5'(rnstgyaxga tnxtrcasrt)3' (n = a, t, g, c; x = n or gap; s = g, c; r = a, g; y = c, t).

Note that the dimeric binding of the LuxR product produces the kind of non-linear concentration/response behavior discussed in [**19**] and widely seen in DNA binding protein transcriptional control. This nonlinear response is an essential element of signal restoration and digital control of expression.

The transcription of the right operon also enhances the production of LuxI, and thus the VAI synthesase, and VAI. We see here the key component of a schmidt-trigger positive feedback gate – once transcription is turned, the enhancement is self-reinforcing, leading to hysteresis in the transfer curve.

4. Engineered Plasmid Constructs

In order to experiment with intercellular communications, we constructed a series of plasmids, and then transformed them into *E. coli* cells. The plamids can be roughly categorized into three groups: preliminary plasmids (Section 4.1), plasmids that transmit the message by catalyzing the formation of autoinducer

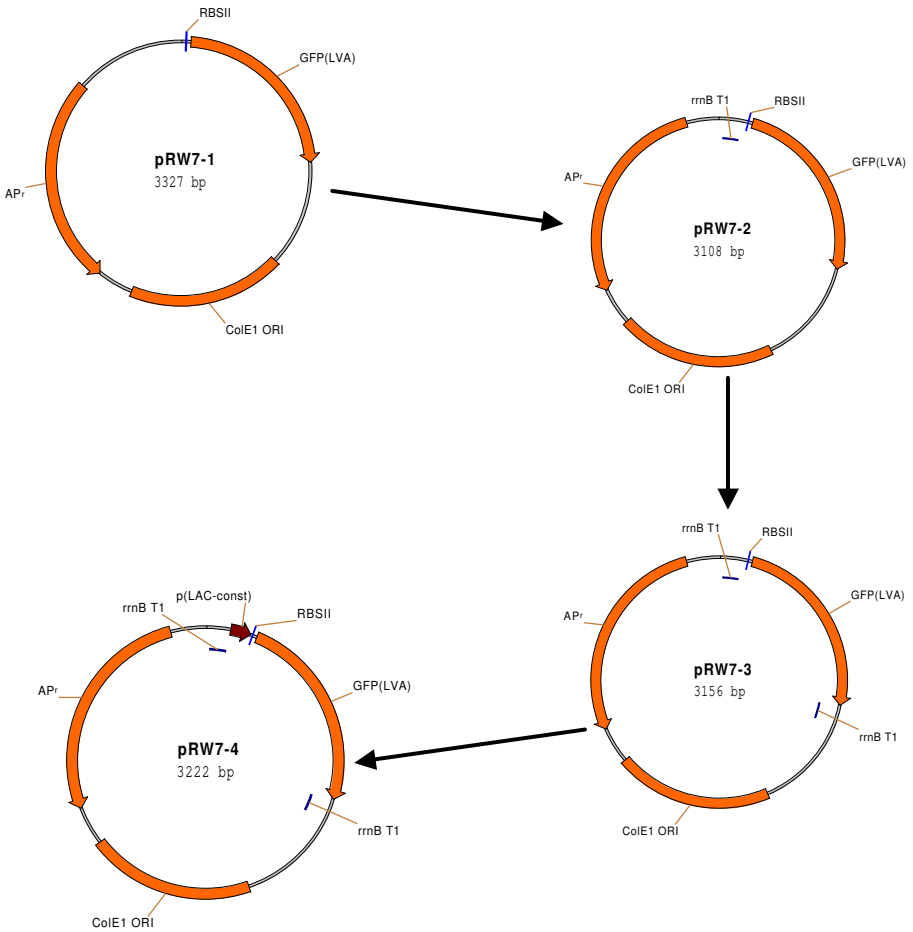

FIGURE 2. Preliminary plasmids

(Section 4.2), and plasmids that respond to the message through the use of the appropriate region of the lux operon (Section 4.3).

4.1. Preliminary Plasmids. Initially, we constructed a series of plasmids (Figure 2) that could serve as templates for cloning the final sender and receiver plasmids. The first plasmid, pRW7-1, combines the backbone of the general purpose high copy number plasmid pUC19 with GFP(LVA) from Clontech pGFP(LVA). Both pUC19 and pGFP(LVA) were digested with SpeI and XmaI, and the GFP(LVA) CDS and its associated synthetic ribosome binding site (RBSII) were cloned into pUC19. GFP(LVA) is a variant of the green fluorescent protein with a destabilizing tail (amino acids RPAANDENYLVA) that results in a protein half life of approximately 40 minutes.

Next, to produce pRW7-2, a transcription termination region (rrnB T1) based on a sequence from pKK232-8 [**25**] was cloned into pRW7-1 using two oligonucleotides. The oligos were annealed by incubating @97°C for 10 minutes, then incubating @65°C for 15 minutes, incubating @24°C for 15 minutes, and finally

storing @4°C, to produce the following double stranded segment with overhangs that match an AatII and XmaI digest:

```
ACCCGGGAATTCCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTC GTTTTATCTGTTGTTTGTCGGTGAACGCTCTCACCGGT
TGCATGGGCCCTTAAGGGTCCGTAGTTTATTTTGCTTTCCGAGTCAGCTTTCTGACCCGGAAAG CAAAATAGACAACAAACAGCCACTTGCGAGAGTGGCCAGCC
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The annealed oligos were then ligated into pRW7-2 digested with AatII and XmaI. The plasmid pRW7-3, which includes the same transcription termination region but on the 5' end of GFP(LVA), was constructed in a similar fashion. The oligos used have HindIII and XbaI overhangs, and were cloned into a pRW7-2 HindIII/XbaI digest.

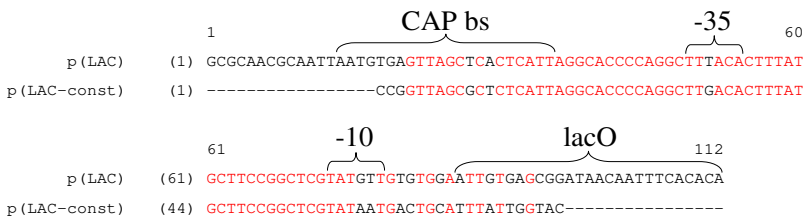

FIGURE 3. Comparison of p(LAC) with p(LAC-const)

The final preliminary plasmid pRW7-4 includes p(LAC-const), a new constitutive synthetic promoter, in front of the GFP(LVA) cds. We designed the constitutive promoter p(LAC-const) based on the LAC promoter, as shown in Figure 3. In p(LAC-const), the lacO and CAP binding sites have been removed, and the -10 and -35 regions have been modified to resemble the consensus -10 and -35 regions respectively [**22**]. p(LAC-const) was introduced into pRW7-3 (digested with AgeI/Acc65I) using a pair of oligos with AgeI and Acc65I overhangs that were annealed using the same procedure as above. The plasmid pRW7-4 was transformed into *E. coli DH5α* chemically comptetent cells. The construct consisting of p(LAC-cons) followed by GFP(LVA) was verified by detecting the fluorescense of the cells (data not shown).

4.2. Senders. We isolated individual components of the *Vibrio fischeri* system for further use. Plasmids described in this section are shown Figure 4. The LuxI coding region was cloned and placed under control of the Lac promoter of the pUC19 plasmid. This was done by PCR of the pTK1 plasmid DNA using selected primers which included non-matching 5' EcoRI cut sites. Specifically, we performed a PCR reaction using forward primer 5'(agg↓aattcgaataaacgcaagggag)3' and reverse primer 5'(ccg↓aattccctataatatacttag)3', yielding the full length LuxI coding sequence, including the ribosomal binding site, but with paired, distal EcoRI cut sites. PCR was performed using Life Technology High Fidelity PCR Supermix (25μl), 1μl of each primer, and 1μl of $300ng/\mu l$ pTK1 plasmid DNA. The reaction was denatured 5 minutes @94°C, followed by 30 cycles of denaturing 30 seconds @94°C, annealing 30 seconds @50°C, and extension 1 minute @70°C. Reaction products were verified by gel electrophoresis, and separated from primers using the Bio101 Geneclean spin protocol. The purified PCR product was digested with

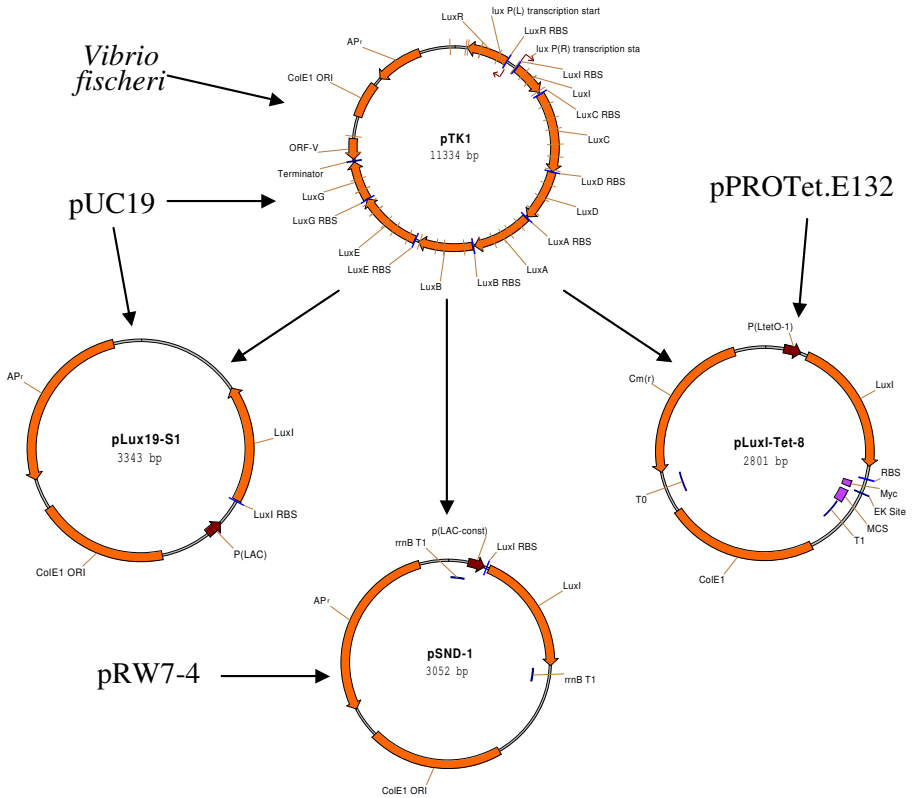

FIGURE 4. Sender plasmids

EcoRI, and ligated with prepared pUC19 vector, which had been EcoRI cut and dephosphorylated with Amersham shrimp alkaline phosphatase.

The resulting ligation was transformed into *E. coli DH5α* and plated on LB AMP. The transformed colonies exhibited two distinct morphologies, clear, small colonies and opaque, large colonies. Six of each colony morphology were streaked, grown, and minipreped. Restriction digests and gel eletrophoresis showed that the small colonies contained the LuxI gene in the correct, expressing, orientation. One such clone, pLuxI19-S1 was chosen for further study.

The same EcoRI digested LuxI PCR product was also similarly cloned into the Clonetech pPROTET.E332 plasmid. This plasmid contains a Col-E1 ori, chlomamphenicol resistance gene, and a TetO controlled promoter. The TetO promoter is inhibited by the TetR gene product, in the presence of the anitbiotic tetracycline. The TetR gene is chromosomally carried in a special version of *E. coli*, which also carries the spectinomycin resistance gene. As a first step, the ligation reaction was transformed into subcloning efficiency DH5α cells, grown up in LB chloramphenicol ($50\mu g/ml$). After verification of the correct insert, miniprep DNA was re-transformed into the TetR containing strain, which was then grown in LB spectinomycin chloramphenicol broth.

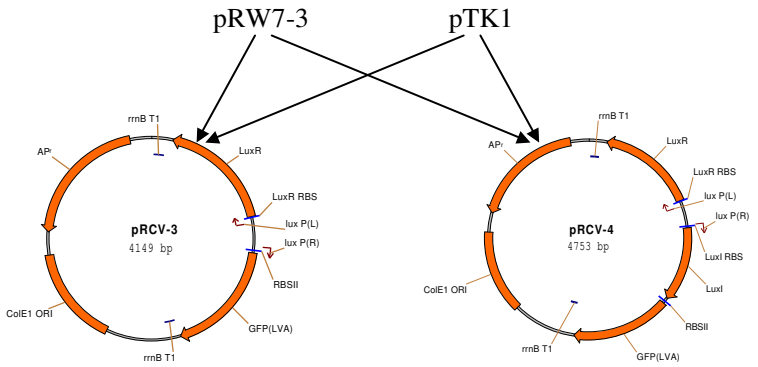

FIGURE 5. Receiver plasmids

The PROTet system allows controlled expression of the inserted gene using varying amounts of a non-growth-inhibitory version of tetracycline, anhydro-tetracycline (aTc). In this way, we can control expression of the LuxI gene, and hence the level of VAI, in these cells, through control over the aTc concentration.

The plasmid pSND-1 was constructed for constitutive expression of luxI, by removing the GFP(LVA) CDS from pRW7-4 and replacing it with luxI from pTK1. A PCR reaction using forward primer 5'(catgggtacctccggaataaagctttacttacgtac)3' and reverse primer 5'(catgaagcttaacaacattaatttaagactgc)3' yielded the luxI coding sequence, including the ribosomal binding site. The PCR product was then ligated with a pRW7-4 Acc65I/HindIII digest, and transformed into chemically competent *DH5α* .

4.3. Receivers. The receiver plasmid pRCV-3 was constructed using pRW7-3 as the plasmid backbone and by inserting the luxR$P_L P_R$ region from pTK1 upstream of GFP(LVA). We performed a PCR reaction using forward primer 5'(catgggtacctccggaataaagctttacttacgtac)3' and reverse primer 5'(catgggtaccggccggtttattcgactataacaaacc)3', yielding the luxR$P_L P_R$ region with Acc65I cut sites at both tails. The PCR product was then ligated into a pRW7-3 Acc65I digest, and the resulting colonies were screened by restriction mapping and and partial plasmid sequencing to ensure that the insert was in the correct orientaion.

The receiver plasmid pRCV-4 served as a control plasmid to verify the ability of the lux operon to exert positive control on the synthesis of GFP(LVA). The luxR$P_L P_R$luxI region from pTK1 was extracted with a PCR reaction using forward primer 5'(catgggtacctccggaataaagctttacttacgtac)3' and reverse primer 5'(ccttggtaccggccgaacaacattaatttaagactgc)3'. As above, the PCR product was then ligated into a pRW7-3 Acc65I digest, and the resulting colonies were screened by restriction mapping and partial plasmid sequencing to ensure that the insert was in the correct orientaion.

5. Intercellular Signalling Experiments

5.1. Sending a constant cell to cell signal. Our first intercellular communications experiment involved the sending of a constant signal from one cell type to another. Cultures of *E. coli* containing the pRCV-3 plasmid and the pSND-1 plasmids were grown overnight @37°C in LB AMP. A 96 well clear bottom plate was

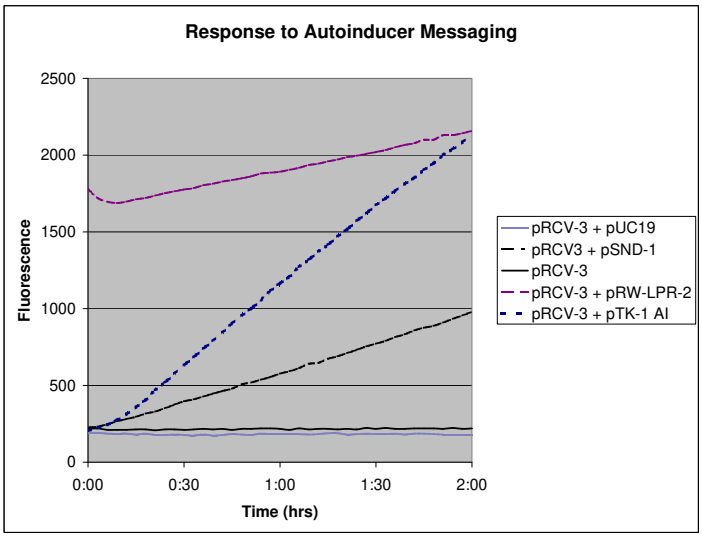

FIGURE 6. Verification of communication constructs

loaded with 200μl of LB AMP in each well. 10μl of pSND-1 cells were loaded horizontally to each cell, along with controls consisting of constitutive GFP expressing cells, pUC19 containing *E. coli* and a series of wells containing extracted VAI (see below).

Vertically, 10μl of cells containing the pRCV-3 construct were also loaded into each well. Thus, each well contained a variety of senders, and a uniform set of receivers. The plate was grown in a Biotek FL-600 fluroescent plate reader for two hours, and read for fluorescence at the GFP(LVA) peak (excitation filter 485/20 nm, emission filter 516/20 nm). The results are shown in figure 6. Wells containing only the pRCV-3 cells, or with added pUC19 cells, showed no increase in fluorescence. The well containing pRCV-3 cells and pRW-LPR-2 cells (which express GFP(LVA)) served as a positive control. Wells containing the pRCV-3 cells plus extracted pTK1 autoinducer showed high, and increasing levels of fluorescence. Cells with pRCV-3 and pSND-1 showed the expected increase in fluorescence demonstrating successful cell to cell signalling.

5.2. Autoinducer extraction and characterization of the receiver module. The receiver plasmid pRCV-3 was further characterized by inducing the promoter with VAI extracted from cell culture. Cultures of *Vibrio fischeri* and of *E. coli* containing the pTK1 plasmid were grown overnight to stationary phase in GVM broth or LB AMP respectively @30°C which allows evaluation of their bioluminescence. After verification of light production, 100 ml of the cultures were centrifuged at 3300 g, and the supernatent collected. The supernatent was extracted with 10 ml of ethyl acetate by vigorous shaking in a separatory funnel for 10 minutes. The ethyl acetate extract (upper fraction) was separated and dried under vacuum. The resulting crude extract was redissolved in 1ml of DI water to provide 100x VAI extract.

Similar 96 well plate experiments were performed in which we analyzed the effectiveness of serial dilutions of the VAI extracts from pTK1 and *Vibrio fischeri*

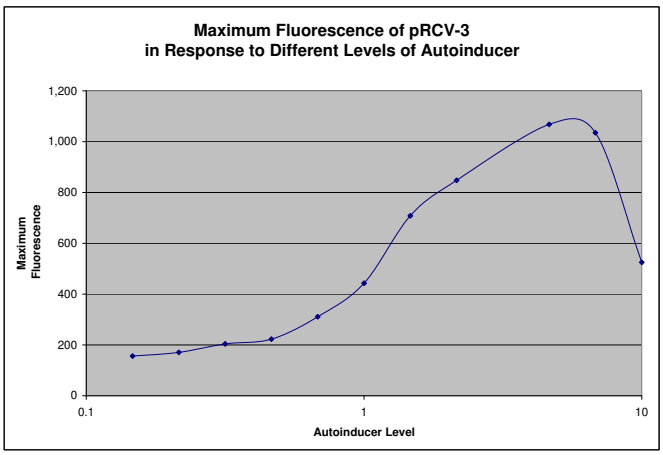

FIGURE 7. The effect of different autoinducer levels on the maximum fluorescence attained.

in inducing GFP expression of the pRCV-3 cells. We found (Figure 7) that both the *Vibrio fischeri* and pTK1 extracts were about equally effective at inducing expression of the pRCV-3 promoter, as measured by GFP production. High levels of the extract, however, were toxic to cells, and resulted in relatively low expression levels.

5.3. Sending controlled cell to cell signals. Finally, we placed the LuxI gene under control of the Tet promoter from the Clontech pPROTet system. The experiment is schematically represented in Figure 8. In one cell, the pLuxI-Tet-8 plasmid exerts controlled expression of the LuxI autoinducer synthesase using the Tet operon. The synthesase catalyzes the conversion of normal cellular metabolic products into VAI; thus, controlling the LuxI expression level controls the VAI production in the cells. The VAI produced within the cells migrates though the cell membrane of the sender, into the culture medium, and through the membrane of the receiver – a cell containing the pRCV-3 plasmid. There, it interacts with the N-terminal domain of the LuxR DNA binding protein product, disabling it from binding to the lux box binding site. The expression of the GFP reporter gene is enhanced, resulting in high levels of fluorescence.

The experiment involved the incubation of similar mixed cell cultures on 96 well clear bottom plates. One important difference was the culture medium – the pPROTET cells carry spectinomycin and chlormaphenicol resistance, while the pRCV-3 cells carry ampicillin resistance. The experiments were carried out by growing overnight cultures of both types of cells in the appropriate antibiotic containing medium, followed by centrifugation at 4000g to remove the medium, and resuspension to similar cell density in LB containing no antibiotics, so that both cell types could grow.

A similar arrangement of horizontally different pLuxTet senders (different colonies from the pPROTet/LuxI ligation reaction) and vertically similar receivers were loaded, including a set of wells containing no senders ("RCV only") and a set of cells containing pTK1 VAI at levels previously shown to induce high level expression.

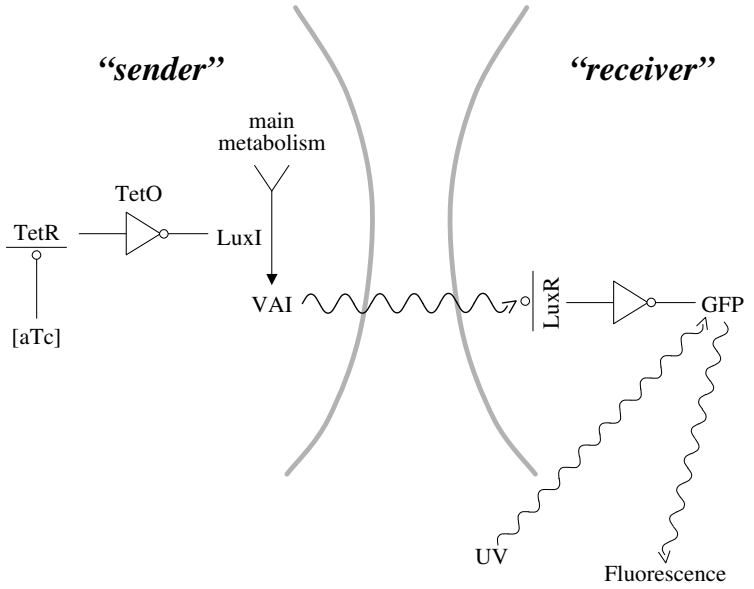

FIGURE 8. Circuit diagram of gradient communications

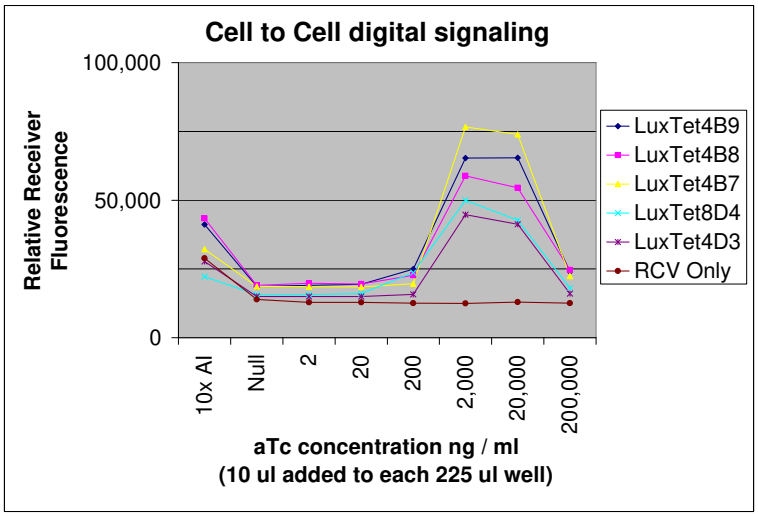

FIGURE 9. The effect on the receiver of transmitting the message at different intensities

Figure 9 shows the results of this experiment after culturing the plate for four hours @37°C. As expected, the null wells showed no enhancement of fluorescence, while the 10x VAI positive control wells exhibited fluorescence. The three experiments labeled LuxTet4B7, LuxTet4B8, and LuxTet4B9 include senders where the pLuxI-Tet-8 plasmid was transformed into BL21-PRO cells, while the experiments labeled LuxTet4D3 and LuxTet4D4 include senders where the pLuxI-Tet-8 plasmid

was transformed into *DH5α* cells.[1] In wells containing sender cells induced with aTc at levels below about $20ng/ml$, only a small fluorescent response is exhibited by the receiver cells. In wells induced with aTc levels above $200ng/ml$, a significant response was observed. Sufficiently high levels of aTc inhibited cell growth.

6. Conclusions

We have successfully isolated an important intercellular communication mechanism from a naturally occurring bacterial system, analyzed its components, and engineered its interfaces with standard genetic control and reporter mechanisms. While we have captured one such communication mechanism, realistic genetically controlled developmental systems will require perhaps dozens of such signals. The LasI/LasR system from *Pseudomonas aeruginosa* [**5**], for example, appears to encode a similar regulatory system, but one which uses a different, and non-cross reacting autoinducer, and a different structure homologous to the lux box. Isolation and characterization of such additional communication mechanisms will allow the construction of more complex multicellular systems.

Appendix A. Extraction and analysis of the lux operon structure from natural constructs

A stab of *Vibrio fischeri* MJ1 was obtained from Fotodyne, Inc. and restreaked and grown @28°C on GVM plates (10g tryptone, 5g Difco casamino acids, 25g NaCl, 4g $MgCl_2$, 1g KCl, 15g agar per liter, pH 7.4) [**31**], and verified for light production. An overnight culture from a single colony was grown with vigorous shaking in liquid GVM medium @28°C to an OD of 2.5.

Genomic DNA was isolated from 100 ml of the overnight culture using a scaled up version of the CTAB procedure [**2**], hooked out of solution, ethanol reprecipitated, and dissolved in pH 8.0 TE 10:0.1.

A SalI restriction digest (cut site 5' g↓tcgac 3') of the genomic DNA was performed and run on a 0.8% TAE agarose gel to verify cutting and average fragment length. The digestion was performed in a 20μl reaction volume with 2μl (40 units) of SalI (NEB #138), 2μl of 10x NEBuffer SalI, supplemented with 0.2μl of 100x BSA, with 2μl of genomic DNA ($300ng/\mu l$). The digestion was carried out for 1 hour @37°C, followed by a denaturation @65°C for 20 minutes.

A similar SalI restriction digest of pUC19 plasmid DNA (NEB #304-1, ($1\mu g/l$)) was prepared and verified by 0.8% TAE agarose gel electrophoresis. pUC19 is a high copy number colE1 ori plasmid carrying the ampicillin resistance gene, and the LacZ β-galactosidase gene. Inserts into the pUC19 multiple cloning site disrupt the activity of the LacZ gene, and allow screening for insertions on XGAL plates using blue/white screening.

A ligation overnight @14°C of the SalI digests of pUC19 and the *Vibrio fischeri* genomic DNA was performed. The 20μl ligation reaction contained 2μl (800 Cohesive End Ligation Units) of T4 DNA Ligase (NEB #202), 2μl of 10x NEB T4 Ligase Buffer, 1μl of the SalI pUC19 digest DNA, and varying amounts (0.5, 1, 2, 4, 8μl) of the SalI genomic DNA digest.

[1] In BL21-PRO cells, TetR (needed for controlled induction of the Tet promoter) exists on a plasmid, while in *DH5α* TetR is part of the chromosomal DNA.

The ligation mixture was transformed by 45 second heat shock @42°C into Life Technology subcloning efficiency *E. coli* DH5α [F^- Φ80*d lacZ* Δ(*lacZYA*− *argF*)-*U*269 *deoR recA*1 *endA*1 *hsdR*17 ($r_k^-.m_k^+$) *phoA supE*44 λ− *thi*−1 *gyrA*96 *relA*1] and spread on LB AMP ($50mg/l$) / XGAL ($25mg/l$) / IPTG ($100\mu M$) plates. The plates were incubated overnight @37°C to grow colonies, and evaluated for the optimal vector : insert ratio by inspection of blue/white colony ratios. The optimal ratio ligation mix ($2\mu l$ of genomic DNA in the ligation) was spread onto 20 LB AMP plates, grown overnight @37°C, and further incubated for six hours at room temperature to allow expression of the heat sensitive Lux gene cassette. Plates were visually examined for luminescent colonies following dark adaptation. A single luminescent colony, labelled pTK1, was detected, and streaked out onto LB AMP plates.

A luminescent colony was used to innoculate 200 ml of LB AMP medium, and and overnight culture was grown with vigorous water bath shaking @37°C to OD 3.0. A standard Qiagen spin maxiprep was performed, yielding pTK1 plasmid DNA.

The pTK1 plasmid DNA was digested with EcoRI (as above with 2μl , 20 units, of NEB #101, (cut site 5' g↓aattc 3') and SalI restriction enzymes. The unrestricted plasmid DNA, as well as the restriction digests were run on 0.8% TAE agarose gels, along with samples of pUC19, pUC19 digested with SalI, and a 1 kb Biorad DNA ladder.

pUC19 digests with EcoRI and SalI showed the expected 2.86 kb fragment, while the undigested pUC19 showed several bands presumably corresponding to supercoiled variants of the circular plasmid.

pTK1 digests showed an EcoRI fragment of approximately 11.5 kb and two SalI fragments of sizes 2.7 kb and 9 kb. The 2.7 kb fragment was identified as the double cut pUC19 vector. The 9 kb fragment was identified as the luminescence causing vector insert.

The insert of pTK1 was sequenced with the Sanger dideoxy technique using ABI Bigdye terminator ready reaction mix and primer walking. Initial sequences were primed with the M13 -47 forward primer 5'(cgccagggttttcccagtcacgac)3' and the M13 -48 reverse primer 5'(agcggataacaatttcacacagga)3'. Subsequent primer sequences were determined by choosing 18-22 mers from about 500 bp into the previous sequence. Primer sequences were chosen with approximately 50% gc content, typically ending (3') with two or more gc bases to act as clamps. Reverse complement sequences were also chosen from about 250 bases into the new sequence using similar criteria, to prime a reverse direction verification sequence. The sequencing reactions were carried out in a 25μl volume, containing 4μl of ABI BigDye Ready Reaction Mix, 4μl of Sequencing buffer (200mM Tris-HCl, 5mM $MgCl_2$, pH 9), 1μl of primer, and 1μl of pTK1 plasmid template. The sequencing reactions were run on an MJ PTC-200 thermal sequencer, with a program consisting of a denaturing step of @95°C for 10 minutes, followed by 30 cycles consisting of 10 seconds @94°C, 5 seconds @50°C, and 4 minutes @50°C. Sequencing reactions were then held until use @4°C in the cycler. The sequencing reaction mix was gel filtered in a Princeton Separations sepharose column, dried in a Speedvac, and resuspended in 25μl of ABI template suppression buffer. After vortexing and spin down, the resuspended product was denatured @95°C for 1 minute, and snap cooled on wet ice. The product was transfered to septum covered tubes and inserted into the ABI 310 sequencer. Samples were run with a 61 cm capillary, filled with ABI POP-6

sequencing gel, held @50°C, and with a 3KV, 60 second capillary injection, followed by a 12.2KV 120 minute electrophoresis run.

Sequences were proofread with ABI Sequence Manager software, and several omitted bases of the initial sequences were manually corrected. Corrected sequences were assembled using the ABI Autoassembler software, yielding a complete sequence which was again proofread. Two additional sequences were run to verify questionable sequence calls. The final insert was determined to be 8654 bases long, and has been submitted to GENBANK as accession AF170104 [**21**].

The resulting sequence was compared against other known sequences in the GENBANK NR database, and found to be essentially identical to previously reported sequences for the *Vibrio fischeri* MJ-1 strain, for those portions which had been previously sequenced. Specifically, the reported sequence is completely identical in those regions reported in GENBANK M25751 [**7**] for strain MJ-1, and differing only slightly from the earlier reports from sequencing this strain in GENBANK Y00509 [**13**]. Specifically, these differences are a missing triplet gtt at base 891, a short, recovered, frame shift mutation at bases 904 - 910, and a substituted cgc for a gcg triplet at base 1137. Given the identical sequence reported in M25751, these differences probably represent mutations in their copy of the original MJ-1 sequence.

Related *Vibrio fischeri* strains show high homology in these areas as well. Entry M19039 [**6**], sequencing from ATCC 7744 (type strain) shows 23 point mutations relative to our sequencing of this area. M96844, [**17**], sequenced from the squid symbiot ES114 strain, shows a relatively distant but still quite close homology.

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