Anne Condon
Grzegorz Rozenberg (Eds.)

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DNA Computing

6th International Workshop on DNA-Based Computers, DNA 2000
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Preface

The papers in this volume were presented at the 6th International Meeting on DNA Based Computers, organized by the Leiden Center for Natural Computing and held from June 13 to June 17, 2000 at The Lorentz Center, University of Leiden, Leiden, The Netherlands. DNA Computing is a novel and fascinating development at the interface of computer science and molecular biology. It has emerged in recent years, not simply as an exciting technology for information processing, but also as a catalyst for knowledge transfer between information processing, nanotechnology, and biology. This area of research has the potential to change our understanding of the theory and practice of computing.

The call for papers and poster presentations sought contributions of original research and technical expositions in all areas of bio-computation. A total of 33 abstracts were submitted of which 16 were accepted for presentation and included in the proceedings. The papers were selected by the program committee based on originality and quality of research and on relevance to the bio-computing field. Invited talks were given by Masami Hagiya (Tokyo University), Laura Landweber (Princeton University), John Reif (Duke University), Thomas Schmidt (Leiden University), and Lloyd M. Smith (University of Wisconsin). Invited papers based on the talks by Hagiya and Reif are included in this volume, along with the contributed papers. Additional tutorials were held on the first and last days of the conference.

The conference was held under the auspices of the ACM Special Interest Group on Algorithms and Computation Theory (ACM SIGACT) and the European Association for Theoretical Computer Science (EATCS). We gratefully acknowledge support and sponsorship from the following organizations: the European Molecular Computing Consortium (EMCC), the European Commission (EC) Institute for Programming research and Algorithmics (IPA), the Leiden Institute of Advanced Computer Science (LIACS), the Lorentz Visitor Center (LC), and the Netherlands Organization for Scientific Research (NWO).

The program committee wishes to thank all those who submitted papers for consideration.

March 2001

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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 [1,3,4,24,26,29,30] has looked at some of these robustness and pattern formation issues in simulation, with intriguing results. We

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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.

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 [11,16,19,23,29] 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, agri-

culture, 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

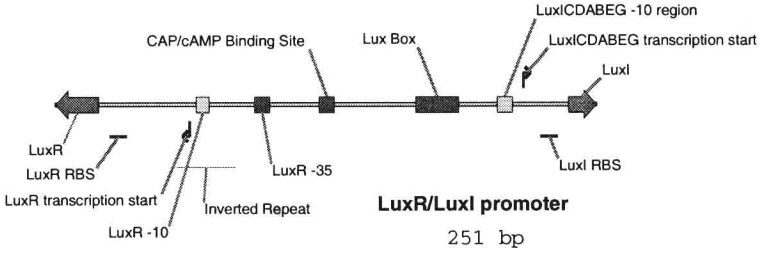

Fig. 1. LuxR and LuxI promoter regions from *Vibrio fischeri.*

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 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 palindromic 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 nonlinear concentration/response behavior discussed in [19,29] 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 plasmids can be roughly categorized into three groups: preliminary plasmids (Section 4.1), plasmids that enable cells to transmit the message by catalyzing the formation of autoinducer (Section 4.2), and plasmids that enable cells to 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 CAAAATAGACAACAAACAGCCACTTGCGAGAGTGGCCAGGCC
```

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.

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

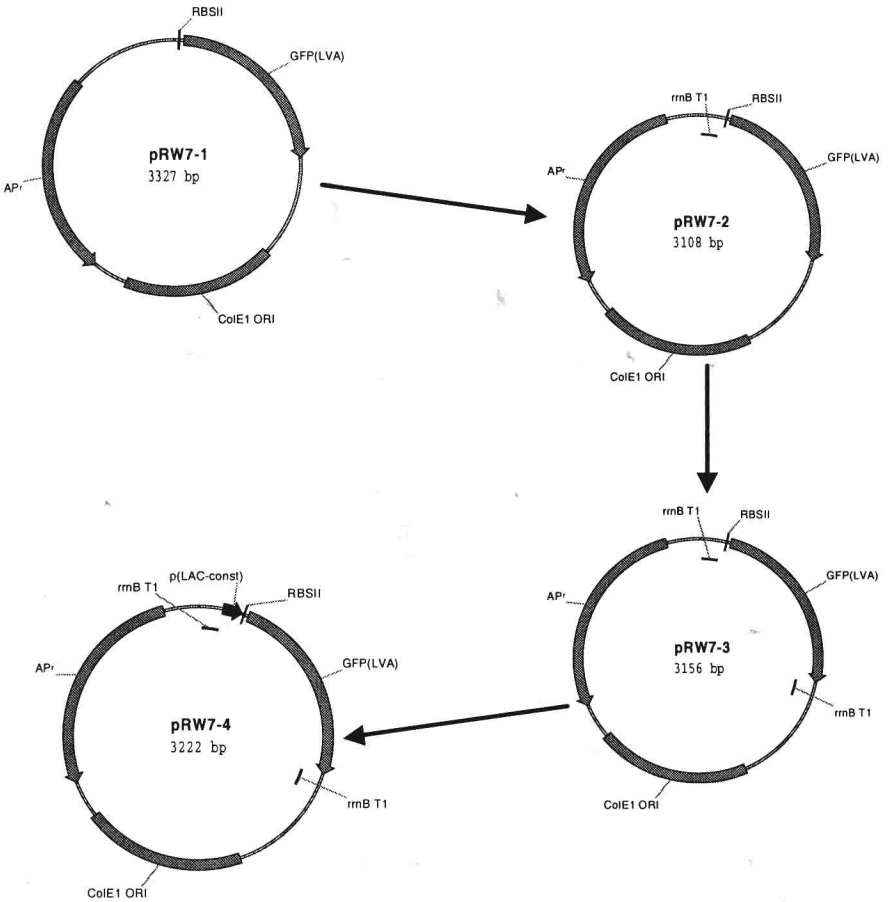

Fig. 2. Preliminary plasmids

```
                        1          CAP bs                    -35          60
p(LAC)          (1) GCGCAAGGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTAT
p(LAC-const)    (1) ----------------CCGGTTAGCGCTCTCATTAGGCACCCCAGGCTTGACACTTTAT

                       61         -10          lacO                 112
p(LAC)          (61) GCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACA
p(LAC-const)    (44) GCTTCCGGCTCGTATAATGACTGCATTTATTGGTAC----------------
```

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

that were annealed using the same procedure as above. The plasmid pRW7-4 was transformed into *E. coli DH5α* chemically competent cells. The construct consisting of p(LAC-cons) followed by GFP(LVA) was verified by detecting the fluorescense of the cells (data not shown).

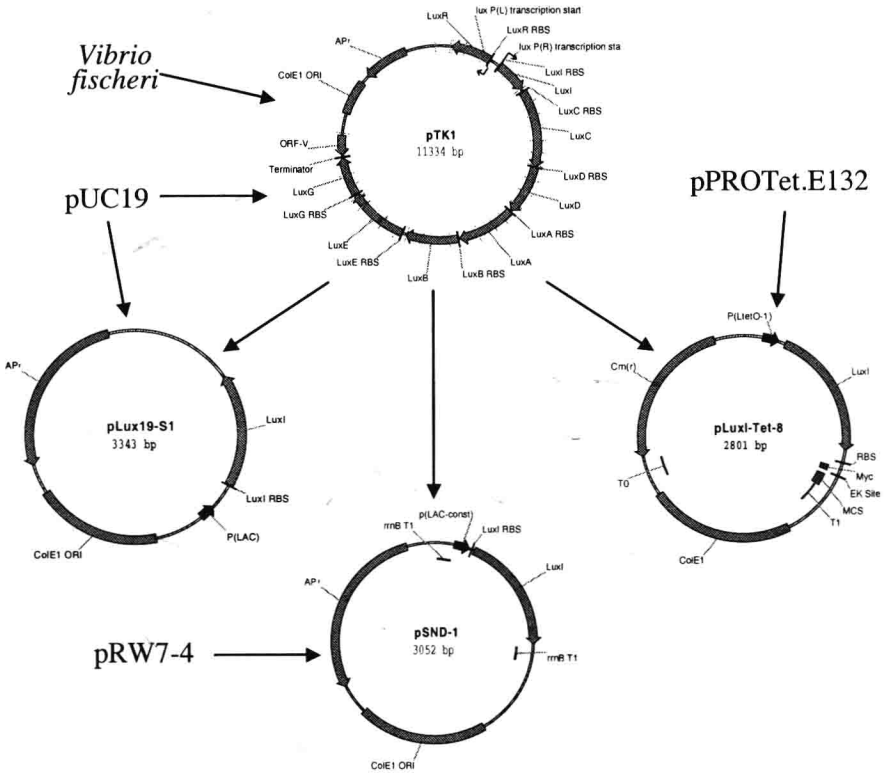

Fig. 4. Sender plasmids

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 300$ng/\mu l$ 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 EcoRI, and ligated with prepared pUC19 vector, which had been cut with EcoRI and dephosphorylated with Amersham shrimp alkaline phosphatase.

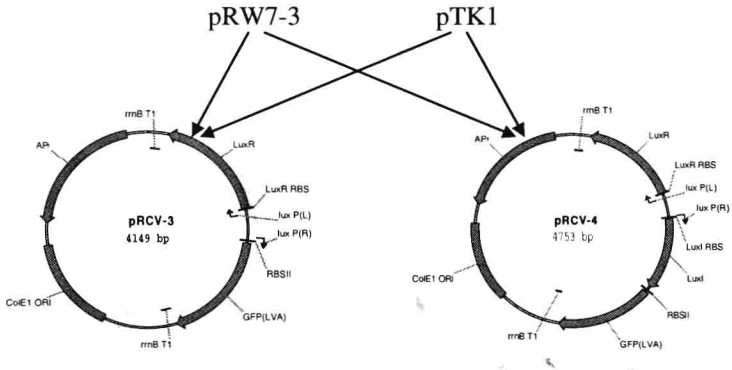

Fig. 5. Receiver plasmids

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.

The PROTet system allows controlled expression of the inserted gene using varying amounts of a non-growth-inhibitory version of tetracycline, anhydrotetracycline (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 luxRP_LP_R region from pTK1 upstream of

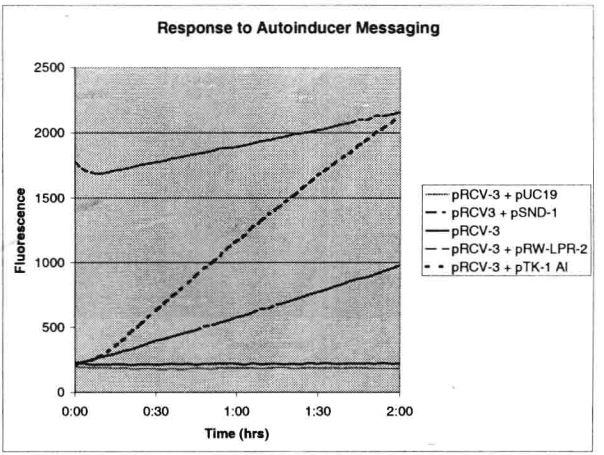

Fig. 6. Verification of communication constructs

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 DH5α* containing the pRCV-3 plasmid and the pSND-1 plasmids were grown separately overnight @37°C in LB AMP. A 96 well clear bottom plate was 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 cells expressing GFP constitutively, *E. coli DH5α* containing pUC19, 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 fluorescent plate reader

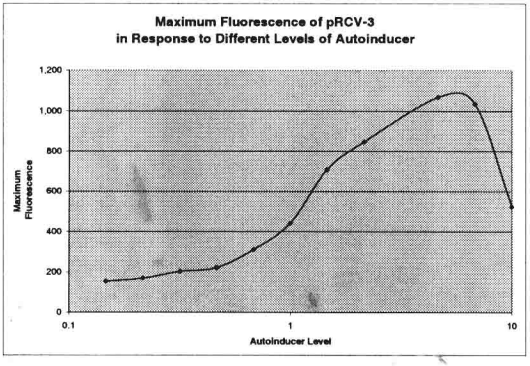

Fig. 7. The effect of different autoinducer levels on the maximum fluorescence attained.

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 supernatant collected. The supernatant 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.

We performed experiments to analyze the effectiveness of serial dilutions of the VAI extracts from pTK1 and *Vibrio fischeri* in inducing GFP expression of the pRCV-3 cells. Both the *Vibrio fischeri* and pTK1 extracts were about equally effective at inducing expression of the pRCV-3 promoter, as measured by GFP production. Figure 7 shows that increasing levels of autoinducer yielded increasing GFP expression by the receiver. High levels of the extract, however, were toxic to the cells, and resulted in relatively low fluorescence 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 Fig-