Experimental Methods in Support of the Development of a Computational Model for Quorum Sensing in 
*Vibrio fischeri*

Yann S. Dufour

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of 
Master of Science in 
Biology

APPROVED

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Keywords: *Vibrio fischeri*, quorum sensing, gene regulation, luminescence, microfluidics, mathematical modeling

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Department of Biology

Abstract

The quorum sensing signaling system based on intercellular exchange of N-acyl-homoserine lactones is used by many proteobacteria to regulate the transcription of essential genes in a signal density-dependent manner. It is involved in a number of processes including the development of highly organized bacterial communities, e.g., biofilms, the regulation of expression of virulence factors, production of antibiotics, and bioluminescence. The extensive genetic and biochemical data available on the quorum sensing system in *Vibrio fischeri* allows the development of a systems biology approach to undertake a spatial and dynamical analysis of the regulation throughout the population. The quorum sensing regulated lux genes are organized in two divergent transcriptional units: luxR and luxICDABEG. The latter contains the genes required for luminescence and the luxI gene necessary for synthesis of an N-acyl-homoserine lactone commonly called autoinducer (AI). The luxR gene codes for a transcriptional regulatory protein that activates the transcription of both operons at a threshold concentration of AI. The positive feedback loop induces a rapid increase of transcription level of the lux genes when a critical population density is reached (reflected by the concentration of AI in the environment). With a combination of molecular biology tools, physiological analysis, and mathematical modeling we identified critical characteristics of the system and expect to assign parameter values in order to achieve a comprehensive understanding of the dynamics. An ordinary differential equation mathematical model is used to investigate the dynamics of the system and derive parameter values. In parallel a novel microfluidic cell culture experimental set-up is used to carefully control environmental parameters as well as to achieve chemostatic conditions for high-density cell populations. An unstable variant of the green fluorescent protein was used as a reporter to follow the time response at a single cell level. Thus spatial organization and noise across the population can be
analyzed. Plasmids carrying different genetic constructs were transformed in a recombinant *Escherichia coli* strain to specifically identify genetic and biochemical elements involved in the regulation of the *lux* genes under diverse conditions. Then the quantitative data extracted from batch culture and microfluidic assays were used to assign parameter values in the models. The particular question being investigated first is the nature of the regulation to increasing concentration of the signal. The hypothesis tested is that the regulation of the production of the signal by individual cells is biphasic and, therefore, quorum sensing should be robust to global and local variations in cell density.
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Chapter One: Literature Review

**Quorum sensing and biofilm formation**

Recent identification of quorum sensing systems in a number of proteobacteria has led to an increased interest in this model of cell-density dependent regulation of gene expression (32, 43, 74). First discovered in Gram-negative bacteria as playing a role in the regulation of bioluminescence in *Vibrio fischeri* (55), gene and protein homology studies have since led to the discovery of very similar mechanisms among a large group of proteobacteria (32, 43, 50). In this group of organisms, quorum sensing seems to be the underlying mechanism that regulates many communal behaviors and allows the elaboration of complex cell communities. Indeed, it has been found involved in the formation of structures like biofilms, the synchronization of virulence factor expression, the synthesis of antibiotics, and the production of bioluminescence (32, 43, 50).

Such coordination among single cells requires gene regulation mechanisms in response to one or more signals. In Gram-negative bacteria the signaling compounds are small diffusible molecules belonging to the family of N-acyl-homoserine lactones. These molecules, termed autoinducers (AI), are produced by the cells, and either diffuse through (38) or are transported across the membrane (63). The autoinducer interacts with a dedicated intracellular receptor protein of the LuxR family to form a complex that binds DNA to activate or repress gene transcription (80). Activation of transcription of the *lux* operon occurs via a series of steps (the cooperative binding of AI with LuxR leading to dimerization, the binding on the *lux* box and the positive feedback on transcription of both *luxR* and *luxI*) that makes activation rapid but only when a threshold concentration of autoinducer is reached. The N-acyl-homoserine lactones used by the different proteobacteria vary by the lengths of the acyl side chain and by the active groups present (32, 50).

*Pseudomonas aeruginosa*, an opportunistic plant and animal pathogen that frequently infects lungs of immunodeficient human patients, is widely used as a model to study relationships between pathogenesis, communal behavior and quorum sensing. It relies on a quorum sensing system comprised of multiple N-acyl-homoserine lactone signals to regulate phenotypes critical for virulence and biofilm structure formation (13-
Density-dependent control provides many advantages for the cells. It allows synchronization of the expression of extra cellular virulence factors at the moment when the impact on the environment would be the most significant. In the scenario where a pathogen wants to invade a host, it is in its best interest to wait for a critical population mass to be reached before alerting the host defense system when expressing virulence factors. A synchronized attack from a large population has a better chance to overwhelm the host defenses and establish infection. Mutants of *P. aeruginosa* defective in quorum sensing signaling were severely diminished in their ability to infect mice, although they grew at the same rate as the wild-type strain in rich laboratory medium (61, 66, 67, 78). To establish a successful long-term infection, *P. aeruginosa* cells work together to form a community harboring a complex architecture and dynamical behavior. The community undergoes several steps to produce a mature biofilm that can be several hundred micrometers thick. They embed themselves in a polysaccharide extracellular matrix and grow in mushroom-shaped structures, creating channels through the biofilm for nutrient and waste diffusion. This architecture gives to the community a great resistance against host immune response or antibiotic treatments (19). The involvement of the quorum sensing system in the biofilm formation is not fully characterized yet, but mutants of *P. aeruginosa* deficient in cell signaling showed biofilms with greatly affected structures. As a consequence, they became more susceptible to antibiotic treatments (19). Many other examples of biofilm formation correlated to pathogenesis were described recently in studies with other bacterial strains (33, 42, 44, 59, 72).

The formation of such self-organized sophisticated architectures and the accompanying dynamical behavior necessitates a distributed population control based on only local information processed by each individual cell. Therefore the quorum sensing system is a primary target in the effort to control or disrupt formation of such communities. A new generation of antimicrobial agents could emerge from molecules that inhibit quorum sensing. Several targets are considered in order to disrupt cell-to-cell communication. First, various analogs of \(S\)-adenosyl methionine (which is involved in N-acyl-homoserime synthesis) such as \(S\)-adenosylhomocysteine, \(S\)-adenosylcysteine, and sinefungin, have been identified as inhibitors of N-acyl-homoserime lactone synthesis in *P. aeruginosa* (60). Second, some bacteria have been identified to produce enzymes that specifically degrade N-acyl-homoserime lactones (18, 40). Although the ecological
function is not well-understood, the enzymes have drawn a lot of attention from the medical field. Finally, the receptor itself (the LuxR-type protein family) can be targeted. The red algae, *Delisea pelchra*, has been characterized to synthesize N-acyl-homoserime lactone analogs (halogenated furanones) that interact with CarR from *Erwinia carotovora* to produce an inactive complex (45, 46). In addition, other analogs have been tested in vitro for their inhibitory activity (45, 46, 68, 75, 76, 86).

**The quorum sensing system in Vibrio fischeri**

*V. fischeri* was isolated from the light organs of marine fishes and squids but it is capable of free-living in seawater (65). It is housed in the animal light organ as a symbiont and produces light in this environment. The light produced is used by squid hosts to mask their shadows from predators at night. *V. fischeri* adapts to the light organ environment upon colonization by changing gene expression. For instance, it increases a transporter of branched-chain amino acids on its surface. It is believed that it is with these amino acids that the squid maintains the bacterial population. The bacteria and the hosts exchange signals, but one of the mechanisms used by the *V. fischeri* population to recognize and respond to its localization in the host light organ is the quorum sensing system based on N-acyl-homoserime lactone signal (49). Actually, quorum sensing was first discovered in *V. fischeri* because it is directly linked to light emission, which is easy to observe. The genes required for the density-dependent regulation and all the *lux* genes required for light production can be isolated in one DNA fragment and easily cloned (29).

The quorum sensing gene regulation in *V. fischeri* is one of the simplest and best understood systems to date. The quorum sensing regulated *lux* genes are organized in two divergent transcriptional units: *luxR* and *luxICDABEG* (28) (Figure 1.1). The latter contains the genes required for the production of light and the *luxI* gene necessary for synthesis of the diffusible signal autoinducer molecule N-3-oxohexanoyl-L-homoserine lactone. The *V. fischeri* membrane is permeable to the autoinducer and no transport mechanism is necessary to allow diffusion into the extracellular environment (23). The *luxR* gene codes for a transcriptional regulatory protein that is active in the presence of autoinducer and begins activating the transcription of both operons when a threshold concentration of autoinducer is reached. This positive feedback mechanism leads to a rapid increase in the transcription level of the *lux* genes resulting in light production at
high cell densities (32, 74). At low cell density although a basal level of transcription is maintained, the level of autoinducer produced by LuxI is too low to build up a high concentration of autoinducer in the environment and allow the efficient formation of the LuxR-AI complexes.

The binding site for the LuxR protein on the DNA, termed the lux box, is situated at the -42.5 position relative to the luxI transcription start site and consists of a 20 base pair palindrome (16, 24, 26). A sequence homologous to the lux box is also found in the luxD gene (11 of the 20 base pairs are identical). It offers a second putative binding site for LuxR but with a weaker affinity than the lux box and is suspected to act a regulatory element in the transcription of luxR (69). Other global factors known to be involved in the overall regulation of the lux operon regulation, include GroESL, FNR, CRP, LexA, LuxO, LitR and oxygen or iron limitations, but they were ignored in this study (1, 20, 21, 31, 50). Experiments were carried in chemostatic conditions and the quorum sensing system was transferred into recombinant Escherichia coli host where most of these additional levels of regulation are not present.
Figure 1.1. Cartoon model illustrating the quorum sensing signaling system components in V. fischeri. (not to scale). The proteins LuxI, which synthesizes the 3-oxo-C6-homoserine-lactone molecules (autoinducers), and LuxR, the receptor, are synthesized at a basal level at low cell density. The autoinducer diffuses freely through the cell membrane. At high cell density, the concentration of autoinducer is high and the LuxR-AI complexes form to bind to the lux box. A positive feedback mechanism occurs in which transcription of both luxR and the lux operon is increased. This mechanism leads to a rapid amplification of AI production and initiation of bioluminescence as parts of the quorum sensing response. The LuxR-AI complex can bind weakly on a region in luxD, the luxD box, and may induce a negative feedback on the system at very high concentration of AI.
**Development of mathematical models for quorum sensing systems**

Although questions remain, the *V. fischeri* quorum sensing system is the best understood of the more recently discovered similar systems in proteobacteria. Therefore it is a natural starting point to develop quantitative models to study the quorum sensing mechanisms. Only a few quorum sensing models have been proposed to date. An extremely simplified deterministic model for *V. fischeri* (36) and a similar, but more detailed model for *Pseudomonas aeruginosa* (17), predicted that synthesis of autoinducer and the response from a dynamical system defined by two stable states (basal and high production of autoinducer). In both model the conclusion reached was that the switch from one state to another is triggered by an accumulation of the extracellular autoinducer. Hysteresis (resistance to a change of state) of the system, both upon induction or down-regulation, results from the positive feedback mechanism on autoinducer synthesis. Two other models addressed the population response resulting from quorum sensing, trying to define the parameters affecting the critical quorum number (56, 81). These theoretical models all focused on the onset induction of the system and the definition of a quorum. Cox et al. (12) undertook an analysis of noise in gene regulation and its effect upon timing of induction of the system. They concluded that noise triggers induction earlier but is not a fundamental characteristic of the quorum sensing system. Yet, none of the models proposed focused on the system behavior further than initial induction. The quorum sensing often induces phenotypes that are sustained for a relatively long time by bacterial communities even through changing environmental conditions (e.g. biofilms and symbiosis). The mechanisms that would provide robustness and adaptability to the control of autoinducer synthesis and the dependent gene regulations are proposed to be investigated theoretically.

The advantages of mathematical models are that simulations can be run by placing the virtual bacteria in conditions not easily achievable in laboratory experiments but observed in natural environments (e.g. in lungs, or in the light organs of squids). Specific variables can be manipulated to observe the consequences and better identify the underlying mechanism of regulation in the actual cells. Nevertheless, before a theoretical
model can be reliable it has to be based on demonstrated hypotheses tested experimentally.

A computational model of the *lux* operon autoregulation has been proposed by Dr. Andre Levchenko at Johns Hopkins University to describe quantitatively the mechanism underlying quorum sensing. The novel hypothesis proposed by Dr. Levchenko states that the regulation of the production of autoinducer by individual cells is biphasic in nature and therefore makes quorum sensing robust to global and local variations in cell density.

The model proposed is an algorithmic, bottom-up approach that takes the natural noisiness and variability into account in the analysis of experimental data. The virtual cells are seeded randomly on a matrix according to a predetermined density value. The production level of autoinducer of each cell in response to the local autoinducer concentration follows a response curve that will be characterized experimentally according to the population average. The diffusion of autoinducer produced from each cell through the matrix is simulated by a symmetric neighborhood in which the autoinducer concentration value changes linearly from the highest level at the center (the cell) to zero. The size of the neighborhood is then determined by the level of autoinducer production of each cell, and this level is dynamically updated according to the local autoinducer concentrations. Figure 1.2 represents the response curves that have been arbitrarily determined to simulate the population responses in term of autoinducer production according to the two mechanisms of regulation hypothesized: down-regulated (biphasic) and non-down-regulated (monotonic) quorum sensing responses.

![Figure 1.2](image)

**Figure 1.2.** AI autoregulation curves assumed in Dr. Levchenko’s model. (biphasic (A) and monotonic (B) regulation curves). AI production values correspond to the intracellular AI concentration achieved at the corresponding extracellular AI level (arbitrary units).
Figure 1.3 is a plot of the resulting average autoinducer production per cell and the average autoinducer concentration in the population according to the response curve considered. While down-regulation of *luxR* has previously been examined experimentally (69, 70), the down-regulation of the *lux* operon has never been considered before. But it is clear from the simulations that there is, as a result, a dramatic difference in terms of energy expenses to maintain the quorum sensing response and the total autoinducer concentration in the population. It is believed that behavior resulting from down-regulation affects also the population tolerance to local variations in cell numbers therefore equalizing the response across a spatially non-homogenous cell distribution.

**Figure 1.3.** Simulations from Dr. Levchenko’s model. The data plotted are the average of 5 different runs of the simulation for random cell distributions for each value. (A) represents the average autoinducer production per cell corresponding to Fig 1.2A. for the solid line (no down-regulation) and Fig 1.2B. for the dashed line (with down-regulation). (B) represents the average autoinducer concentrations on the matrix corresponding to Fig 1.2A. for the solid line (no down-regulation) and Fig 1.2B. for the dashed line (with down-regulation).
**Microfluidic systems**

Microfluidic devices allowing the manipulation of liquids in channels having dimensions on the order of ten micrometers is a new technology still under development. They were primarily designed for chemical and biochemical miniaturized analytical tools (e.g. genomics and proteomics) and biomedical devices (e.g. *in vivo* drug delivery), but the versatility of plastic polymer based devices is drawing large enthusiasm from very diverse fields. Early systems were fabricated by etching glass or silicon, which is difficult and expensive. However, new fabrication techniques have greatly reduced the costs of the devices, which add to the advantages of reduction of reagent consumption and portability. Polydimethylsiloxane (PDMS) is viewed as a promising material for microfluidic device fabrication because it presents many advantages. It is relatively inexpensive, channels can be created by molding or embossing (which are easier than etching) with a high fidelity on the micron scale. Furthermore it is optically transparent (for an extensive review on device fabrication see (48).

At the micron scale, aqueous flows are laminar (no turbulences mix the fluids) and this is taken as an advantage to create flow patterns or chemical gradients. The combination of this characteristic with the intricate architecture that is possible in the design of the channels enables the creation of an environment with a complex geometry and a precise control over the environmental parameters (5, 37, 83, 84). No attempt has been made to develop microfluidic devices as an analytical tool for bacterial culture studies yet. Previous work was mainly aimed at cell sorting (30, 47). A novel microfluidics-based method is being developed by Dr. Andre Levchenko and Dr. Alex Groisman (at UC San Diego) to study bacterial population behavior in a spatially defined environment, and more particularly, the quorum sensing response at a single cell level with variations of local autoinducer concentration.
The green fluorescent protein as a reporter

Unfortunately, the reporter based on light production by the luciferase enzyme gives a signal too low to be easily detected in a single cell (79). Therefore as a part of this project, new genetic constructs will be developed using unstable variant forms of the green fluorescent protein (GFP) gene to replace production of light by fluorescence. GFP was initially extracted and purified from the jellyfish, *Aequorea victoria*. It fluoresces when excited with blue light (maximally at 395 nm with a minor peak at 470 nm) emitting a brilliant green light (peak emission at 509 nm) and is easily detectable with epifluorescence microscopy (7). The protein became a very successful reporter through the past ten years because it can be expressed in a wide range of organisms, including *E. coli*, *Caenorhabditis elegans*, and *Drosophila melanogaster* (7, 35). It is comprised of 238 amino acids (27 kDA) and the fluorophore is formed by an autocatalytic cyclization of three internal amino acid residues. Maturation requires only the presence of oxygen and normally does not interfere with the host metabolism (10). Nevertheless, in its native form GFP has a long maturation time and is very stable and not degraded by the cells. As a result the protein accumulates in the cell preventing the study of transient expression. Consequently, variant forms of GFP have been developed by site directed mutagenesis to obtain GFP variants that fold more efficiently in *E. coli* (11). The excitation wavelength was also shifted to 488 nm to obtain a better light emission. These variants were then subjected to further development to increase their sensitivity to *E. coli* proteolysis and thus exhibit dramatically reduced half-lives (2, 3). Creating transcriptional fusions of these GFP variants with a DNA promoter region of the *lux* quorum sensing system will allow the study of transient response of single cells to the quorum sensing signal.
**Purpose**

The specific goals of this project were to verify experimentally the computational model of biphasic dependence of the *lux* operon transcription on the intracellular autoinducer concentration, to construct the plasmids encoding unstable variants of GFP with the regulatory sequence of the *lux* operon, and to produce the response curves from the entire population when grown in the presence of different amounts of autoinducer (Chapter 2). This information was correlated to the initial studies done in the microfluidic devices performed in Dr. Levchenko’s lab at the Johns Hopkins University and will help further advance of the mathematical models (Chapter 3). A deterministic mathematical model is also presented to investigate the theoretical role of the *lux* box in the regulation of the quorum sensing system and to initiate an effort to define kinetic parameters characterizing the system (Chapter 4).
Chapter Two: Experimental Analysis

Introduction

The experimental (genetic and physiological analysis) part of the project was driven by two purposes. First, physiological analyses were conducted on the regulation of the quorum sensing system to produce evidence confirming or reputing the biphasic nature of the regulation at high concentrations of autoinducer as proposed by Dr. Andre Levchenko. High autoinducer concentrations are produced when the cell population reaches very high densities ($10^{10}$ to $10^{11}$ cells/ml) (34); in the squid light organ for *Vibrio fischeri*, or in biofilms for *Pseudomonas aeruginosa*. Since liquid batch cultures cannot reach comparable density (usually $10^9$ cells/ml), the autoinducer was provided exogenously. At the same time a genetic analysis of the *lux* operon was undertaken to identify potential elements involved in the transcription regulation as a function of the autoinducer concentration. The primary target was a region homologous to the *lux* box the binding site for the LuxR activator protein, found in the *luxD* gene. It has been previously described to affect *luxR* gene regulation (69) and is suspected to induce a biphasic regulation of *lux* operon transcription.

Second, in order to characterize the system regulation at the single cell level, a fluorescent reporter was substituted for light production. The light production resulting from expression of the *lux* genes can be detected from cell cultures but is too weak for single cell analysis. Consequently green fluorescent protein was considered a good alternative for the reason that it allows a noninvasive detection of the levels of transcription and does not need any substrate to give a strong signal. Unstable variants of GFP were selected with the expectation to be able to follow transient regulation of the quorum sensing system (3).

All of the physiological assays were performed on bacterial batch cultures. The response of the entire population was determined when grown in the presence of different amounts of autoinducer. Cell level resolution experiments done in the microfluidics devices are presented in Chapter Three.
Materials and Methods

Characterizing the biphasic dependence of lux operon transcription on the intracellular autoinducer concentration in *Vibrio fischeri*

To determine the response of the wild-type quorum sensing system, assays were done with *Vibrio fischeri* MJ211 strain (39) (Table 2.1) that contains a nonpolar mutation in luxI. The strain was cultured overnight in Sea Water Complete medium (tryptone 5 g/L, yeast extract 3 g/L, glycerol 3 mL/L, 0.3M NaCl, 0.5X of Instant Ocean® (Aquarium Systems, Mentor, OH), pH=7) at 30ºC before it was subcultured in fresh medium to an OD$_{600}$ of 0.05 and divided into several identical cultures in which known quantities of a racemic mixture of autoinducer 3-oxo-hexanoyl homoserine lactone were added. When the cultures reached an OD$_{600}$ of approximately 0.5 while incubating at 30ºC, the luminescence of 5 µl samples from each culture was measured in a Turner 20/20 luminometer for 4 seconds to determine light outputs.

Characterizing the biphasic dependence of lux operon transcription on the intracellular autoinducer concentration with genetic constructs in recombinant *E. coli*

A recombinant *E. coli* strain JM109 (85) (Table 2.1) was transformed with pJR551 (22) and pSC300 (8) (Table 2.2). pJR551 is a P15A replicon plasmid conferring chloramphenicol resistance that contains the complete lux operon with a nonpolar temperature sensitive mutation in luxI and MudI1681 transposon insertion that inactivates luxR and allows the use of β-galactosidase as a reporter of the luxR promoter. LuxR is provided by pSC300, a ColE1 replicon plasmid conferring ampicillin resistance with luxR under the control of the isopropylthiogalactoside (IPTG) inducible tac promoter. The cells were grown overnight in Luria-Bertani (LB (NaCl 5 g/L, tryptone 10 g/L, yeast extract 5 g/L, pH=7)) medium supplemented with chloramphenicol (30 µg/ml) and ampicillin (100 µg/ml) at 31ºC while shaking. Then they were subcultured to an optical density at 600nm (OD$_{600}$) of 0.05 in the same medium and conditions and grown to early exponential phase (OD$_{600}$ of 0.1). The culture was then subdivided and known quantities of autoinducer, 3-oxo-hexanoyl homoserine lactone, were added to each subculture. After
two hours of incubation at 31°C, the cultures reached an OD₆₀₀ between 0.4 and 0.6 and luminescence outputs were measured before placing the cultures on ice. Luminescence was measured with 200 µl of culture on a microtiter plate in an LUCY 1 luminometer (Anthos Labtec Instruments, Salzburg, Austria) over a 1 second integration time. The β-galactosidase assays were measured with the Tropix chemoluminescent reporter assay kit (Tropix, Bedford, Mass.) and light output was measured in a LUCY 1 over a 30 seconds integration time on 10 µl of the cultures as previously described (24). The luciferase assays were performed with 5 µl of the cultures as previously described (79) and light output was measured in a Turner 20/20 luminometer (Turner Designs, Sunnyvale, CA).

To assay the level of expression in *E. coli* VJS533 transformed with pHV200I- (62), the same growth conditions were used except that the incubation was done at 30°C. LB medium supplemented with ampicillin (100 µg/ml) was used and induction with autoinducer, 3-oxo-hexanoyl homoserine lactone, were performed as described above. Luminescence was measured with 200 µl of culture on a microtiter plate in an LUCY 1 luminometer over a 1 second integration time.

**Table 2.1. Bacterial strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM105</td>
<td>thi rpsL(Str⁻) endA sbcB15 sbcC hsdR4 (rrK₄ mK₄) Δ(lac-proAB) F' [traD36 proAB⁺ lac⁺ lacZΔM15]</td>
<td>(85)</td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F' [traD36 proAB⁺ lac⁺ lacZΔM15]</td>
<td>(85)</td>
</tr>
<tr>
<td>VJS533</td>
<td>ara Δ(lac-proAB rpsL Φ80 lac ZΔM15 recA56</td>
<td>(73)</td>
</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MJ211</td>
<td>MJ-1, Nxr, ΔluxI (~250bp nonpolar deletion)</td>
<td>(39)</td>
</tr>
</tbody>
</table>
Table 2.2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAT213A</td>
<td>pSC300 with an alanine substitution at the position 213 in LuxR</td>
<td>(79)</td>
</tr>
<tr>
<td>pAT216A</td>
<td>pSC300 with an alanine substitution at the position 216 in LuxR</td>
<td>(79)</td>
</tr>
<tr>
<td>pAT229A</td>
<td>pSC300 with an alanine substitution at the position 229 in LuxR</td>
<td>(79)</td>
</tr>
<tr>
<td>pGFI</td>
<td>Ap(^{r}); pBBR1-luxR-luxI-RBS-gfpmut3(^{-})-T(_{0})</td>
<td>This study</td>
</tr>
<tr>
<td>pHV200I</td>
<td>Ap(^{r}); 8.8kb Sall fragment containing the <em>V. fischeri</em> ES114 lux regulon with a nonpolar mutation in <em>luxI</em> in pBR322</td>
<td>(62)</td>
</tr>
<tr>
<td>pJBA88</td>
<td>Ap(^{r}); pUC18Not-luxR-P(<em>{lux})-RBSII-gfpmut3(^{-})-T(</em>{0})-T(_{1})</td>
<td>(2)</td>
</tr>
<tr>
<td>pJBA89</td>
<td>Ap(^{r}); pUC18Not-luxR-P(<em>{lux})-RBSII-gfp(ASV)-T(</em>{0})-T(_{1})</td>
<td>(2)</td>
</tr>
<tr>
<td>pJBA111</td>
<td>Ap(^{r}); pUC18Not-P(<em>{A1/04/03})-RBSII-gfp(LVA)-T(</em>{0})-T(_{1})</td>
<td>(3)</td>
</tr>
<tr>
<td>pJR551</td>
<td>Cm(^{r}); pACYC184 with luxR::Mu(_{A(c, nerAB)}) d11681 (<em>lacZYA</em>, Km(^{r})) and luxICDABEG with a nonpolar point mutation in <em>luxI</em></td>
<td>(22)</td>
</tr>
<tr>
<td>pJE202</td>
<td>Ap(^{r}); 8.8kb Sall fragment containing the <em>V. fischeri</em> MJ1 lux regulon in pMB1</td>
<td>(28)</td>
</tr>
<tr>
<td>pJWP02</td>
<td>Km(^{r}); pBBR1-luxR-luxI-RBS-gfp(LAA)-T(_{0})</td>
<td>This study</td>
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<td>pKE206</td>
<td>pSC300 with an alanine substitution at the position 206 in LuxR</td>
<td>(25)</td>
</tr>
<tr>
<td>pLUZ02</td>
<td>Ap(^{r}); pSP417, luxR-luxI upstream of a promoterless lacZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pLUZ03</td>
<td>Ap(^{r}); pSP417, luxR-luxICD (with luxD box) upstream of a promoterless lacZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pLUZ04</td>
<td>pLUZ03 without the luxD box sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pLUZ05</td>
<td>Ap(^{r}); pSP417, luxR-luxICDABEG(^{-}) upstream of a promoterless lacZ gene</td>
<td>This study</td>
</tr>
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<td>pLV206</td>
<td>pLVA01 with an alanine substitution at the position 206 in LuxR</td>
<td>This study</td>
</tr>
<tr>
<td>pLV213</td>
<td>pLVA01 with an alanine substitution at the position 213 in LuxR</td>
<td>This study</td>
</tr>
<tr>
<td>pLV216</td>
<td>pLVA01 with an alanine substitution at the position 216 in LuxR</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA01</td>
<td>Ap(^{r}); pUC18Not-luxR-P(<em>{lux})-RBSII-gfp(LVA)-T(</em>{0})-T(_{1})</td>
<td>This study</td>
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<tr>
<td>pLVA02</td>
<td>Ap(^{r}); pUC18Not-luxR-luxI-RBSII-gfp(LVA)-T(<em>{0})-T(</em>{1})</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA03</td>
<td>Ap(^{r}); pUC18Not-luxR-luxICD(^{-})(with luxD box)-RBSII-gfp(LVA)-T(<em>{0})-T(</em>{1})</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA04</td>
<td>pLVA03 without the luxD box sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA12</td>
<td>Ap(^{r}); pUC18Not-luxR-luxI-luxC RBS-gfp(LVA)-T(<em>{0})-T(</em>{1})</td>
<td>This study</td>
</tr>
<tr>
<td>pPROBE-AT(^{-})</td>
<td>Km(^{r}); pBBR1-MCS-RBS-gfp-T(_{0})</td>
<td>(51)</td>
</tr>
<tr>
<td>pPROBE(^{-})-gfp[LAA]</td>
<td>Km(^{r}); pBBR1-MCS-RBS-gfp(LAA)-T(_{0})</td>
<td>(51)</td>
</tr>
<tr>
<td>pSC300</td>
<td>Ap(^{r}); luxR cloned in pKK223-3</td>
<td>(8)</td>
</tr>
<tr>
<td>pSP417</td>
<td>Ap(^{r}); pRS415 with a MCS upstream of a promoterless lacZ gene</td>
<td>(64)</td>
</tr>
</tbody>
</table>
Development of strains allowing single cell level resolution analysis using unstable variants of GFP as a reporter

To study the effect of different lux operon elements several constructs were cloned with the unstable GFP(LVA) variant. GFP(LVA) is the most unstable variant readily available and is preferred to study transient gene expression resulting from quorum sensing regulation (3).

First, the 1.02-kb EcoRI-SphI fragment resulting from a partial digestion of pJBA89 (containing the lux operon promoter region and a functional luxR gene) was cloned into pJBA111 replacing the 0.3-kb EcoRI-SphI fragment containing the LacI-repressible promoter to create pLVA01 (Figure 2.1).

pLVA02 (Figure 2.1) was created by first PCR amplifying a SphI-SphI fragment covering the N-terminal region of luxR, the promoter region, the entire luxI gene on pJE202 with primers LuxrscpI (5’-TGGTTAAATGGAAAGTGACAG-3’) and LuxirsbamhI (5’-GCATGGCTCATAGTTAATTCTCTCCTTGGATCCCTTAATATTC TTCACTTTTCATCGTTG-3’) (restriction sites in bold, ribosome binding site (RBS) in italic) which integrates the RBS sequence of gfp(LVA) and a BamHI site. The fragment produced was then cloned into SphI digested pLVA01. After checking the orientation of the cloned fragment and sequencing the junction between luxI and gfp(LVA), the reporter was transformed in E. coli JM109. pLVA12 is similar to pLVA02 but the luxI-luxC intergenic region was conserved to fuse the gfp(LVA) gene directly to the wild-type luxC RBS. A new downstream primer LuxrbsphI (5’-GCATGGCTCATATTCTTCCCTTATAAA TATACTTAG-3’) was designed to position the start codon of gfp(LVA) where the start codon of luxC would be. The SphI-SphI PCR amplified fragment was cloned into SphI-SphI digested pLVA01 in the correct orientation.

A construct equivalent to pLVA02 but with a stable green fluorescent protein variant (52) was made with a vector based on pBBR1 which is considered to be maintain in medium copy number in E. coli. The EcoRI-BamHI fragment from pLVA02 was inserted into pPROBE-AT’ (51) to create pGFI. Joshua Williams in Dr. Stevens’ lab made pJWP02 in the same manner using pPROBE’-gfp[LAA] as the vector; encoding the unstable variant GFP(LAA).
Figure 2.1. Schematic drawing of plasmid constructs with selected restriction sites (not to scale). Abbreviations: ori, origin of replication; lacZ’, truncated lacZ gene; bla, ampicillin resistance gene; RBSII, synthetic ribosome binding site; gfp[LVA], gene encoding Gfp[LVA]; T₀, transcriptional terminator from phage lambda; T₁, transcriptional terminator from the rrnB operon of E. coli. Note: pLVA03 and pLVA04 differ only by a small deletion in the luxD gene. Unique restriction sites are indicated in blue.
Creating reporters to investigate the effects of the *luxD* box in the transcription regulation of the lux operon

To address the effect of a sequence homologous to the *lux* box found within the *luxD* gene, two plasmids differing only in the deletion of this sequence were created (Figure 2.2). A fragment containing the N-terminus of *luxR*, *luxI*, *luxC* and part of *luxD* was excised from pJE202 with *Xba*I and *Bfu*AI restrictions enzymes. *Bfu*AI cuts in the middle of the *luxD* box sequence upstream of its recognition site. A linker DNA sequence was created to reestablish the missing part of the *luxD* box sequence (with the *Bfu*AI restriction site) and create a *Bam*HI overhang (LinkLuxDBoxplus 5’-ATCATTTTGCAGGTCTTTAAG-3’, LinkLuxDBoxminus 5’-GATCCTTTAAAGACCTGCAAAAAT-3’) (restriction sites in bold, stop codon underlined) to clone the whole fragment in *Xba*I-*Bam*HI digested pLVA02 and create pLVA03 (Figure 2.1). pLVA04 was constructed similarly but the linker DNA sequence did not restore the *luxD* box sequence (nor the *Bfu*AI restriction site) (LinkLuxDplus 5’-ATCCTTAATAGTGAG-3’, LinkLuxDminus 5’-GATCCTCACTATTAG-3’).

**pLVA03**

5’-...AGAAGAATGGATCATTTCGAGGTCTTTAAGGATCC…-3’

*luxD* box  *Bfu*AI site  *Bam*HI site

**pLVA04**

5’-...AGAAGAATGGATCATAAGGATCC…-3’

*Bam*HI site

**Figure 2.2.** Sequences of DNA at the junction of the *lux* operon fragment and the vector in both pLVA03 and pLVA04. They illustrate the small deletion made in the *luxD* box from pLVA03 to pLVA04. The *luxD* box sequence is indicated in italics, the restriction sites are in bold and the stop codons are underlined.
Creating reporters to investigate the transcription regulation of the *lux* operon using β-galactosidase as a reporter

To address the validity of the green fluorescence protein as a reporter for *lux* operon transcription activity the *lux* operon fragments from pLVA02, pLVA03 and pLVA04 were cloned in the vector pSP417 (64) containing the *lacZ* gene. The β-galactosidase protein encoded by *lacZ* has been widely used a reporter in *E. coli* due to its robust activity.

The *Eco*RI-*Bam*HI fragments from pLVA02, pLVA03 and pLVA04 were cloned in pSP417 digested with *Eco*RI and *Bam*HI to create pLUZ02, pLUZ03 and pLUZ04 respectively. pLUZ05 was created by cutting pJE202 with *Xba*I and *Nco*I to extract a fragment containing the full *lux* operon and cloning it into pSP417 digested with *Xba*I and *Bam*HI with a linker from *Nco*I to *Bam*HI (Link*Nco*IBamHIplus 5’-CATGGAATTAATC-3’, Link*Nco*IBamHIminus 5’-GATCCATTTATGTT-3’) (stop codon in italic). *Nco*I cuts within the *luxG* gene. Therefore the terminator sequence of the *lux* operon was not included and a stop codon was inserted in the linker.

Characterizing the transcription regulation of the *lux* operon in GFP reporters in response to exogenous autoinducer concentration

To assay the transcription regulation as a function of the autoinducer concentration, *E. coli* JM109 was transformed with pLVA01 and pJBA88 separately. The two *E. coli* mutant strains were grown overnight in LB medium supplemented with ampicillin (100 µg/ml) at 30°C while shaking. Then they were subcultured to an optical density at 600 nm (OD\(_{600}\)) of 0.025 in the same medium and conditions and grown to early exponential phase (OD\(_{600}\) of 0.1). The culture was then subdivided and known quantities of the autoinducer, 3-oxo-hexanoyl homoserine lactone, were added to each subculture. After two hours of incubation at 30°C, the cultures reached an OD\(_{600}\) between 0.6 and 0.8. Fluorescence was measured with 750 µl of culture diluted in 1.5 mL of LB in a single cuvette fluorometer VERSAFLUOR (Bio-Rad Laboratories, Hercules, CA) with an excitation filter at 480 (±20) nm and an emission filter at 510 (±10) nm over a 4 second integration period.
Characterizing the transcription regulation of the *lux* operon with a functional production of autoinducer in *E. coli* using the β-galactosidase protein activity as a reporter

*E. coli* strain JM109 was transformed with pLUZ02, pLUZ03, pLUZ04, and pLUZ05 independently. The mutant strains obtained all contained a functional quorum sensing system with active production of autoinducer 3-oxo-hexanoyl homoserine lactone. To assay the transcription regulation in function of population density, the four *E. coli* mutant strains were grown overnight in LB medium. A temperature of 30ºC and the appropriate selective agent concentration (ampicillin at 100 µg/ml) was maintained through the whole experiment. Then the cells were centrifuged and resuspended in fresh medium to remove the autoinducer present in the medium and they were subcultured to an OD₆₀₀ of 0.005 and grown to early exponential phase (OD₆₀₀ of about 0.3) before the quorum sensing system was induced. The cultures were centrifuged again and inoculated in 5 mL LB medium with a starting OD₆₀₀ at 0.025. Optical density measurements and samples were periodically taken during bacterial growth. The β-galactosidase activity was measured with the Tropix chemoluminescent reporter assay kit (Tropix, Bedford, MA) and light output was measured in a LD400 luminometer (Beckman Coulter, Fullerton, CA) over a 15 seconds integration time on 5 µl of the cultures as previously described (24).

Characterizing the transcription regulation of the *lux* operon with natural production of autoinducer in *E. coli* using GFP as a reporter

*E. coli* strain JM109 was transformed with pLVA02, pLVA12, pGFI, pJWP02, pLVA03 and pLVA04 independently. The mutant strains obtained all contained a functional quorum sensing system with active production of autoinducer 3-oxo-hexanoyl homoserine lactone. To assay the transcription regulation as a function of the population density, the six *E. coli* mutant strains were grown overnight in LB medium. A temperature of 30ºC and the appropriate selective agent concentration (ampicillin at 100 µg/ml or kanamycin at 50 µg/ml) were maintained through the whole experiment. Then the cells were centrifuged and resuspended in fresh medium to remove the autoinducer present in the medium and they were subcultured to an OD₆₀₀ of 0.025 and grown to early
exponential phase ($OD_{600}$ of 0.1) before the quorum sensing system was induced. The cultures were centrifuged again and inoculated in 100 mL LB medium with a starting $OD_{600}$ at 0.005. Samples of 200 µL were taken in triplicate periodically during bacterial growth to measure optical density and fluorescence output. Both measurements were done on 96 wells microtiter plates with an optically transparent bottom (NUNC, Roskild, Denmark) using a SPECTRAFluorPlus (Tecan, Grödig, Austria) plate reader. The absorbance filter was set at 590 nm and the fluorescence filters were set at 485 nm for excitation light and 535 nm light emission detection. Absorbance values were converted to be equivalent to $OD_{600}$ readings. Fluorescence readings were corrected to remove background fluorescence using a culture of $E. coli$ containing pLVA01 grown in LB medium as a reference.

**Characterizing the effects of variant forms of LuxR with impaired Al-LuxR-DNA interaction**

To assay the affect of impaired forms of LuxR on the quorum sensing regulation a series of plasmids derived from pSC300 were introduced in $E. coli$ JM109 together with the reporter pJR551. Those plasmids differ from the pSC300 by only one mutation in the $luxR$ sequence that changes the residue to an alanine at the position 206 (pKE206) (25), 213 (pAT213A), 216 (pAT216A), 229 (pAT229A) in the protein sequence (79).

The cells were grown overnight in LB medium supplemented with chloramphenicol (30 µg/ml) and ampicillin (100 µg/ml) at 31°C while shaking. Then they were subcultured to an optical density at 600nm ($OD_{600}$) of 0.025 in the same medium and conditions and grown to early exponential phase ($OD_{600}$ of 0.1). The culture was then subdivided and known quantities of the autoinducer, 3-oxo-hexanoyl homoserine lactone, were added to each subculture. After two hours of incubation at 31°C, the cultures reached an $OD_{600}$ between 0.6 and 0.8. Luminescence was measured with 200 µl of culture on a microtiter plate in an LUCY 1 luminometer over a 1 second integration time.

The genes coding for selected variants of the LuxR proteins were then introduced in the vector pLVA01 to create pLV206, pLV213 and pLV216 (the alanine substitution is at the position 206, 213 and 216 in $luxR$, respectively). The C-terminus fragment of the $luxR$ gene containing the mutation was PCR amplified from pKE206, pAT213A or pAT216A. The primers used were EcoRiluxr (5'-GAATTCGCGGTTAATTTTTAAA
GTATGGGCAATC-3’ (restriction site in bold, stop codon underlined) and Xbailuxr (5’-CTCGCGATCATTTATCCTCATTC-3’). The fragments were cloned in pGEM-T® (Promega Corporation, Madison, WI) to be sequenced, and then they were excised with EcoRI and XbaI restrictions enzymes and cloned into EcoRI-XbaI digested pLVA01.

To assay the activity of the LuxR variants with the new vectors, *E. coli* JM109 was transformed with pLV206, pLV213 and pLV216 separately. The *E. coli* mutant strains were grown overnight in LB medium supplemented with ampicillin (100 µg/ml) at 30°C while shaking. Then they were subcultured to an OD$_{600}$ of 0.025 in the same medium and conditions and grown to early exponential phase (OD$_{600}$ of 0.1). The cultures were then subdivided and known quantities of the autoinducer 3-oxo-hexanoyl homoserine lactone were added to each subculture. After two hours of incubation at 30°C, the cultures reached an OD$_{600}$ between 0.5 and 0.7. Fluorescence and absorbance were measured on samples of 200 µL from each culture. Both measurements were done on 96 wells microtiter plates with an optically transparent bottom using a SPECTRAFluorPlus (Tecan, Grödig, Austria) plate reader. The absorbance filter was set at 590 nm and the fluorescence filters were set at 485 nm for excitation light and 535 nm light emission detection.
**Results and discussion**

**Initial studies to identify biphasic regulation of lux operon transcription**

Initial studies were done in *Vibrio fischeri* strain MJ211 to characterize the *lux* operon regulation in response to an increasing amount of autoinducer over a range comparable to those estimated to be produced within the light organs of squid hosts (6). This strain contains a single copy of the *lux* operon in the chromosome with a non-polar knockout mutation in *luxI* disabling the endogenous production of 3-oxo-hexanoyl homoserine lactone. The autoinducer was supplied exogenously and the transcription level of the operon was followed through light output from the culture. The light production per cell is not an exact measurement of the *lux* operon transcription activity (regulation may happen at other levels); however it is considered to be a good first estimation. Figure 2.3 represents the light output measured after the culture was exposed to different concentrations of autoinducer. Down-regulation was observed at the highest autoinducer concentrations tested. The autoinducer concentration inducing the maximum light production in this strain is remarkably high compare to what was previously described in the literature. A comparable behavior was found when the assay was done on *E. coli* strain VJS533 transformed with pHV200I (62) in the same range of autoinducer concentrations. Figure 2.4 represents the light outputs measured, and the curve follows the same trend as in Figure 2.3 except the light output is unusually low. The increasing light production by the cultures showed an affect on the rate of growth of the cells (data not shown). To minimize this effect, the starter cultures were grown first in the absence of autoinducer and the exposure time of the cultures to autoinducer was limited to two hours. Therefore, in this time frame, the affect on the rate of growth was minimal (data not shown).

It has been described that LuxR in the presence of high autoinducer concentrations is capable of negatively regulating itself (22). The down-regulation in transcription of *luxR* would induce a down-regulation of the *lux* operon expression. To study the regulation of *luxR* expression and its affect on the quorum sensing regulation, *E. coli* JM109 was transformed with the plasmids pJR551 (22) and pSC300 (8).
Figure 2.3. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in *V. fischeri*. Assays done with *V. fischeri* strain MJ211 cell culture at an OD$_{600}$ of 0.5. Results represent one trial of an assay done in duplicate. (RLU/OD: relative light units per optical density unit).

Figure 2.4. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in *E. coli* pHV200I$. Assays done with *E. coli* strain VJS533 transformed with pHV200I$ cell culture at an OD$_{600}$ of 0.5. Results represent an assay done in duplicate. The arrow bars are representative of the range for the values measured in triplicate. (RLU/OD: relative light units per optical density unit).
This strain expresses LuxR constitutively through the transcription of luxR on pSC300 and the activity of the wild type luxR promoter is reflected by the β-galactosidase output of the β-galactosidase reporter gene inserted in luxR on pJR551. The results represented in Figure 2.5 show that the response to variation of the autoinducer concentration results in a biphasic dependence of the expression of both transcriptional units of the lux operon. Therefore LuxR autoregulation is not the only factor responsible for luxI downregulation. The luciferase assay, which determines the concentration of the luciferase protein, is considered to be an accurate reporter of the lux operon rate of transcription. These results show that the light output curve trend is very similar to the luciferase assay one. Therefore the light output can be considerable a fairly reliable reporter for preliminary studies. In this assay the peak of maximum expression of the lux operon corresponded to significantly lower autoinducer concentration than observed in the previous two experiments. It could be hypothesized that is the result of the overexpression of LuxR, but the same phenomenon is observed even when luxR is controlled by its native promoter (presented in the following chapters).

**Development of a fluorescent reporter to characterize lux operon transcription regulation**

In the prospect of developing a reporter for quorum sensing activity in the microfluidic chamber experiments, the assays with the same range of autoinducer concentrations were done on E. coli JM109 transformed with pJBA88 (2) or pLVA01 (Figure 2.6). The light production was replaced as a reporter for the expression of the lux operon by unstable variants of GFP. These strains will be used to observe the lux operon response at a single cell level with an epifluorescence microscope during the microfluidic chamber experiments. They expressed a response different from that observed in the E. coli JM109 (pJR551 and pSC300) with the transcription induced at about the same concentration of autoinducer but characterized by a monotonic behavior as the autoinducer concentration increased. The lower fluorescence signal expressed from pLVA01 compared to pJBA88 is explained by the fact that pLVA01 produces the variants GFP(LVA) that has a shorter half-life in the E. coli cells than GFPmut3* (2, 11).
Figure 2.5. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in E. coli pJR551 pSC300. Assays were done with E. coli strain JM109 (pJR551 pSC300) at an OD$_{600}$ of 0.5. (A) Luminescence assay on 200 µl culture samples. (B) Luciferase assays on cell extract. (C) β-galactosidase assays on cell extract. The graphs are representative of experiments done in duplicate. Error bars represent the range of readings done in triplicate. (RLU/OD: relative light units per optical density unit).
Figure 2.6. Monotonic response of *lux* operon transcription to an increasing amount of autoinducer. Assays done on *E. coli* strain JM109 transformed with pJBA88 (● blue) or pLVA01 (▲ red) cell culture at an OD$_{600}$ of 0.8. Results represent one trial of an assay done in duplicate. Error bars represent the range of readings done in triplicate. (RFU/OD: relative fluorescence units per optical density unit).
pLVA01 will be used in the microfluidics experiments described in Chapter 3. The autoinducer dependence of the GFP(LVA) expression will be used to investigate GFP(LVA) stability as well as the autoinducer diffusion in the microfluidics devices.

**Investigating the effect of different elements of the lux operon on a functional quorum sensing system in *E. coli* using the β-galactosidase protein activity as a reporter**

Facing difficulties to assay the activity of a functional quorum sensing system in *E. coli* using GFP(LVA), a family of vectors was created using the β-galactosidase protein activity as a reporter. pLUZ02 contains functional luxR and luxI genes that are sufficient to create the predicted quorum sensing response. To investigate the role of the sequence homologous to the lux box present in the luxD gene (termed luxD box), pLUZ03 and pLUZ04 were created almost identical with a deletion of the luxD box sequence in pLUZ04. pLUZ05 contains the whole lux operon and was used as a control for the wild-type phenotype. Figure 2.7 illustrates the activation of the quorum sensing system when the cell population reached a threshold density. The cells transformed with pLUZ02 induced transcription of the reporter between an OD 600 of 0.3 and 0.4 while the cultures containing pLUZ03, pLUZ04 or pLUZ05 induced transcription later in the growth (OD 600 between 0.6 and 0.7). This observation indicates that the genetic elements missing in pLUZ02 have an effect on the system regulation. However it is not a consequence of the absence of the luxD box because no significant difference was observed between pLUZ03 and pLUZ04. Transcription of the reporter in pLUZ05 is weaker than in the other vectors. This is proposed to be the result of a less efficient transcription due to the distant position of lacZ in the transcriptional unit.

It appeared that one or more genetic elements regulate the induction of the quorum sensing system; and those elements would be in the region between luxI and luxD in the operon.
Figure 2.7. Autoinduction of the lux operon transcription during bacterial growth with a β-galactosidase reporter. β-galactosidase assays done on *E. coli* strain JM109 transformed with pLUZ02 (▲ red), pLUZ03 (♦ blue), pLUZ04 (♦ violet) or pLUZ05 (• green) cell culture. Results represent one trial of an assay done in triplicate. (RLU/OD: relative light units per optical density unit).
Development of fluorescent reporters to characterize *lux* operon transcription regulation in the presence of a functional quorum sensing system

In order to study the diffusion of the quorum sensing signal through a cell culture in the microfluidic devices, cells expressing a functional quorum sensing system (synthesis of the signal and receptor) needed to be developed. Hence, GFP was introduced in a series of vectors to report the system activity. Different constructs were made to identify a combination suitable for the microfluidics experiment setup limitations (background fluorescence and signal strength). The vectors pGFI and pJWP02 have the same backbone, both contain *luxR* and *luxI* fused to a *gfp* gene, but pGFI produces a stable green fluorescent protein while pJWP02 produces an unstable variant (GFP(LAA)). pLVA02 and pLVA12 are also homologues, they contain *luxR* and *luxI* fused to an unstable variant *gfp*(LVA) but in pLVA12 the *luxI-luxC* intergenic region was conserved and the translation of *gfp*(LVA) is initiated with the wild-type *luxC* ribosome binding site while pLVA02 contains a synthetic ribosome binding site. *E. coli* cultures transformed with these vectors were grown in batch cultures to follow the quorum sensing regulation in term of cell density. Figure 2.8 illustrates the expression of the various green fluorescent proteins through bacterial growth. pGFI and pJWP02 differ only in the variant of GFP they encode, therefore the quorum sensing system logically induced transcription at about the same cell density (OD$_{600}$ of 0.3). The instability of GFP(LAA) resulted in a weaker signal from the culture as GFP(LAA) got degraded faster. pLVA02 and pLVA12 cannot be directly compared to the other two vectors (they have a different vector backbone from pGFI and pJWP02), but it can be noted that induction happened also at about the same cell density (OD$_{600}$ of 0.3). This observation indicates that the four vectors are functionally equivalent. pLVA02 had a much weaker fluorescence expression than pLVA12 although the ribosome binding site preceding *gfp*(LVA) is suppose to be stronger since it is closer to the consensus sequence. This weak expression could be a result of an inefficient translation initiation or an overexpression of GFP(LVA) leading to misfolding of the protein.
Figure 2.8. Autoinduction of the *lux* operon transcription during bacterial growth with a fluorescent reporter. Fluorescence measurements done on *E. coli* strain JM109 transformed with pGFI (● green), pJWP02 (■ yellow), pLVA12 (▲ orange) or pLVA02 (♦ blue) cell culture. Results represent one trial of an assay done in duplicate. Error bars represent the range of readings done in triplicate. (RFU/OD: relative fluorescence units per optical density unit).
The vectors pLVA03 and pLVA04 contain the same *lux* operon fragments as pLUZ03 and pLUZ04 respectively. It was previously determined that the presence of the *luxD* box do not affect the *lux* operon regulation (Figure 2.7). Figure 2.9 illustrates the expression of the fluorescent reporter through bacterial growth in *E. coli* cultures transformed with pLVA03 or pLVA04. The results are very similar to Figure 2.7 and the conclusions are the same. The two cultures induced transcription at the same optical density which was about 0.7.

Comparison of GFP to the β-galactosidase protein as a reporter shows that fluorescence gave a higher background signal (according to the GFP stability) leading to a less dramatic signal increase upon induction. Furthermore the relative long maturation time and half-life of GFP (even for the unstable variants) reduce the possibility to follow fast transient regulation of the quorum sensing system.
Figure 2.9. Effect of the luxD box on the autoinduction of the lux operon transcription during bacterial growth. Fluorescence measurements done on E. coli strain JM109 transformed with pLVA03 (▲ blue) or pLVA04 (● red) cell culture. Results represent one trial of an assay done in duplicate. Error bars represent the range of readings done in triplicate. (RFU/OD: relative fluorescence units per optical density unit).
Initial study on the effect of deficient LuxR protein variants on the quorum sensing system regulation

An experiment to investigate the affect of LuxR variants on the regulation of the quorum sensing system was done on a two plasmid system. To report the variants activity, light production resulting from the expression of the lux genes from pJR551 was monitored, and the LuxR protein was provided by pSC300 and its derivatives (the pAT-series) (Table 2.2). Several mutants were selected for their impaired abilities to either interact with the RNAP or to bind DNA (79). The results shown in Figure 2.10 coincide with the predictions derived from the data previously published (79) and show different degrees of efficiency of the transcription activation of the lux operon. Higher concentrations of autoinducer are required to initiate the quorum sensing response; consequently the biphasic behavior of the system observed with the wild-type LuxR protein (pSC300) is lost for these variants.

A series of reporter plasmids were created homologous to pLVA01 replacing the wild-type luxR gene by the different genes coding for variants of LuxR (from the pAT-series). pLV206 produces a completely inactive LuxR protein variant, while pLV213 and pLVA216 produce LuxR protein variants that have an impaired ability to bind the lux box (Table 2.2). Figure 2.11 illustrates their ability to initiate transcription of the fluorescent reporter in the presence of autoinducer. The results corroborated the predictions made from the results presented in Figure 2.10.
Figure 2.10. Effects of the introduction of different LuxR variants on the quorum sensing system response. Assays done with *E. coli* strain JM109 transformed with pJR551 and pSC300 (♦ blue) or its derivatives (alanine substitution at position 206 (● red), 213 (▲ orange), 216 (■ yellow) or 229 (ж green). The cultures were at an OD$_{600}$ about 0.8. Results represent one trial of experiments done in duplicate. Error bars represent the range of readings done in triplicate. (RLU/OD: relative light units per optical density unit).

Figure 2.11. Effects of the introduction of different LuxR variants on the quorum sensing system response after exposure to exogenous autoinducer (AI). Assays done with *E. coli* strain JM109 transformed with the plasmids indicated on the graph. Results represent one trial of experiments done in duplicate. (RFU/OD: relative fluorescence units per optical density unit).
Conclusions

Due to the heterogeneity of the vectors used in this study, it is unfortunately difficult to draw definite conclusions about factors involved in the quorum sensing system regulation. However the comparison of the results gives some indications to direct further investigations.

The quorum sensing system is able to exhibit both biphasic and monotonic behaviors. In the E. coli system where the other levels of regulation on the lux operon are absent, the monotonic response happened when luxR was under the control of its wild-type promoter and the lux operon was replaced by the gfp gene. The biphasic response of both the luxR promoter and the lux operon promoter was observed when LuxR was constitutively expressed in presence of the full length lux operon. In the V. fischeri system a biphasic response was observed, but other levels of regulation affect the lux operon regulation. Therefore, no conclusions can be drawn with the data presented here.

It has been observed that when luxR was under the control of its wild-type promoter and the full length lux operon is present (E. coli pHV200I- and V. fisheri MJ211) the system required a higher concentration of autoinducer to induce. Similarly, E. coli cultures transformed with pLUZ03, pLUZ04, pLUZ05, pLVA03 or pLVA04 (they all contain luxR was under the control of its wild-type promoter and at least the first part of the lux operon down to luxD) induced at a higher cell density than when only luxI is present (E. coli transformed with pGFI, pLVA02, pLVA12 or pJWP02). An exact measurement of the autoinducer concentration in the bacterial cultures is needed to correlate these two observations, but it indicates that a genetic element critical in the regulation could be present in the region between luxI and luxD. It has been shown that the luxD box is not responsible for the delay of induction observed.

Down-regulation of luxR was observed, but the hypothesis previously proposed (69) that the luxD box plays a role in its regulation at high autoinducer concentration was not supported. Biphasic regulation of luxR could result in the stabilization of the numbers of LuxR-autoinducer complex inside the cell as the autoinducer concentration increase. Combined with the down-regulation of the lux operon, the quorum sensing system would be more robust regarding cell population density variation. The quorum sensing system
appears highly dynamic; a more thorough analysis of the regulation of the system considering different hypotheses will be presented in Chapter Four.

The goal to develop fluorescent reporters for the quorum sensing system to carry out experiments in the microfluidics devices was achieved. The range of fluorescence levels expressed by the different vectors provided choices and flexibility in the development of the microfluidics experiments design. However the relatively long maturation time and half-life of the different variants of GFP remain a concern in the prospect of studying transient regulation of the quorum sensing system.

The series of plasmids containing variants of the LuxR proteins is expected to be completed by a selection of different variants from the collection made by Trott and Stevens (79). These vectors will be used as a tool to investigate the effects of impaired LuxR proteins on regulation of the quorum sensing system and the consequences on the overall population organization in the microfluidic chambers experiments.
Chapter Three: Microfluidics

Introduction

Efforts to study the role of quorum sensing in cell community spatial organization, especially at high cell density, call for the development of new diagnostic tools. Indeed, conventional ways of growing cell cultures (liquid batch culture, agar plates and chemostats) present critical limitations. Conventional liquid batch cultures do not maintain chemostatic conditions especially at high cell density. The nutrients are quickly depleted forcing the cells to switch from exponential growth to a stationary state. Current chemostatic devices were designed to overcome nutrient limitations or waste accumulation. However to achieve renewal of the medium they often create a convective flow leading to perturbations in the bacterial culture environment. Some bacterial strains have strong surface attachment capabilities allowing the use of flow cells to study complex structure formation on surfaces (e.g. biofilm) with an excellent control on the environment. Unfortunately, not all bacterial strains have such surface attachment abilities.

Our desire to initially focus on the simple quorum sensing system found in *Vibrio fischeri* and to conveniently manipulate it genetically calls for the adoption of recombinant *Escherichia coli*, which poorly attach on surfaces, as our experimental system. Therefore the development of microfluidic devices based on microchip fabrication techniques is proposed to grow the cells in chemostatic conditions while immobilized or trapped in a defined volume to achieve very high-density cultures. Furthermore, this system mimics the natural environment of *V. fischeri* when established inside the light organ of *Euprymna scolopes* where it can reach extremely high density values ($10^{10}$ to $10^{11}$ cells/ml) (34). In addition, microfluidic devices allow a precise control of the environmental conditions (e.g. creation of gradients, time response) and the response of the cell community can be followed through an extended period of time at cell-level resolution (37, 77).

Assays done with single cell resolution are critical for the analysis of a bacterial population, especially in community studies where segregation of the population into subpopulations is critical for the architecture of the structure. Noise and dynamic
regulation of gene expression, signaling networks within microenvironments and community geometry have to be considered (27, 57, 71).

The microfluidics devices used in this study are produced by patterning a silicon elastomer (PDMS, polydimethylsiloxane) to form channels and chambers on a micron scale. Fabrication of channel structure is done by soft lithography. Rapid prototyping (creation of a design with a computer-aided design program) is used to create and print a high resolution photomask to produce a positive relief on a silicon wafer (the master) and replica molding (the prepolymer is cast on the master, cured and peeled off) allows dimensions from 1 to 100 µm in bulk polymer with few limitations on the geometry. The master is used for the fabrication of many replicas. Next, the patterned PDMS is reversibly sealed on a cover glass by curing it at moderately high temperature to form a watertight environment that can withstand pressure up to 15 psi. Devices can be recycled several times with no alteration in their geometry by peeling it off the cover glass and washing it before being resealed. PDMS is relatively cheap, nontoxic, optically transparent down to 280 nm (allowing UV/visible absorbance and fluorescence detection), and permeable to oxygen and small molecules. It creates a perfect environment for the observation of bacterial cultures while maintaining them in chemostatic conditions. (For a review on microfluidic systems fabrication in PDMS see (48)).

The environment created in the microfluidic device overcomes some limitations presented by the classic liquid culture. The cells are confined in a defined environment with negligible perturbation and can reach high density. Mounted on an inverted microscope, observations at a single cell level in visible light or by fluorescence detection are possible in real time.

The work presented in this chapter is a continuation of the work undertaken by Dr. Alex Groisman, at UC San Diego, in collaboration with Dr. Andre Levchenko, at the Johns Hopkins University, to develop microfluidic systems. They developed the chip designs to grow bacterial cells in chemostatic conditions and undertook preliminary studies on cell signal diffusion.
Materials and methods

The microfluidic device characteristics

The microfluidic device was fabricated by Dr. Alex Groisman’s laboratory at UC San Diego from an original design made conjointly with Dr. Andre Levchenko at the Johns Hopkins University.

The dimensions of the chambers formed in bulk PDMS are 100 µm in length, 50 µm in width and 5 µm or 1 µm in height depending on the design adopted. Figure 3.1 represents a picture of one shallow chamber taken with a phase contrast microscope from the top with a few E. coli cells trapped in it. The two pillars in the chamber are necessary to support the ceiling because of the flexibility of PDMS. The channels for medium flow have a section of 50 µm by 10 µm. Chambers are connected to channels by three small slits on both sides. Their height of 0.65 µm prevents E. coli cells from escaping the chambers while facilitating nutrient diffusion. The size of the channels presents much less resistance to flow in comparison to the chamber slits, therefore flow in the chambers is considered null. Ideally the cells are unperturbed and the spatial organization of the signal response can be observed. The microfluidic device used was composed of a couple hundred chambers in rows between channels. The channels are all connected to single outlet and inlet connections. Different designs may have more inlet connections to be able to vary the medium composition.

A second layer was created on the device to accommodate a large channel for water circulation. The water flowing on top of the chambers is connected to a water bath and maintains the device at a desired temperature through heat diffusion.

Preparation of the bacterial cultures

To prepare bacterial cultures for the microfluidic growth assays the cells were grown at 30°C in 5 mL of LB. The appropriate amount of selective agent (either ampicillin (100 µg/ml) or kanamycin (50 µg/ml)) was maintained throughout the experiment. When the culture reached early exponential growth phase (OD$_{600}$ = 0.2) the cells were washed with fresh medium to remove autoinducer from the medium and subcultured. The cultures were then loaded in the syringe in early exponential growth
Figure 3.1. Picture of one chamber from the microfluidic device with *E. coli* cells trapped. The picture was taken with a phase-contrast microscope at a 60X magnification. The dimensions are as indicated in the picture. The height of the chamber is 1 µm, and the height of the slits is 0.65 µm. The two pillars in the center of the chamber prevent the structure from collapsing. The chamber is connected on both sides to channels (visible on the right of the picture) by slits that are smaller than *E. coli* cells. The cells are introduced in the chambers by applying pressure inside the device to temporarily deform the structure and allow the cells to travel through the slits.
phase after another wash. The washing steps were necessary to prevent expression of an excessive amount of GFP in the cells and it permitted the loading of cells into the device with a background level of fluorescence as low as possible.

**The microfluidic device set-up**

The microfluidic device was placed on the inverted microscope and taped to the stage. First a waste syringe containing the bacterial culture in its exponential growth phase \((\text{OD}_{600} = 0.2)\) was connected to the waste outlet of the chip. By applying pressure into the syringe (about 1 psi) the liquid culture was pushed into the microfluidic device until all the channels were filled, but not the chambers and the first part of the inlet channel (to avoid later contamination of the fresh medium). Then the syringe containing fresh LB medium supplemented with the appropriate concentration of selective agent (either ampicillin at 100 µg/ml or kanamycin at 50 µg/ml) was connected to the inlet. The pressure between the inlet and the outlet syringes was equilibrated with no external pressure by matching the liquid levels of both syringes to stop any flow in the channels. At this point the cells were sitting in the channels in proximity to the slits of the unopened chambers. To force the opening of the chambers and allow a small number of cells (between 1 and 20) to get in the chambers the pressure was uniformly raised in the channels from both syringes simultaneously (avoiding any flow) to about 8 psi. The pressure temporarily deformed the PDMS allowing the cells to flow through the slits into the chambers. When the chambers were filled with the desired number of cells the pressure was reduced and the slits and chambers returned to their original dimensions trapping the cells inside. The cells remaining in the channels were washed away by generating a strong flow from the inlet to the outlet using a pressure differential of about 5 psi. Finally the pressure in the inlet was reduced to 1 psi to create a moderate flow of fresh medium in the channels. The exchange of medium between the chambers and the channels allowed the cells to grow in chemostatic conditions even when they reached high density \((10^{11} \text{ to } 10^{12} \text{ cells/mL})\).

A water bath set at 30°C was connected to a temperature control channel integrated in the device on a different layer. The heat diffuses through the PDMS and the glass cover slip to maintain the cells at a constant temperature. The actual temperature of
the medium inside the chamber could not be determined, although the observed growth rate of the cells indicated a temperature close to 30°C.

**Fluorescence photomicrography and image analysis**

Cultures were followed by fluorescence photomicrography with a Nikon Eclipse TE2000-U inverted microscope (Nikon Instruments Inc, Melville, NY, USA) at a 60X magnification. Pictures were taken in intervals of 5 minutes with a Spot RT Monochrome CDD camera mounted on the microscope (Diagnostic Instruments Inc, Sterling Heights, MI, USA). The excitation filter was set at 550 nanometers to permit detection of green fluorescence. The exposure time was tailored to the cell strain used in order to avoid saturation by a strong signal at high cell density. The relative total fluorescence value was calculated for each image by summing the intensity of each pixel in the images. The resulting combined intensity values were plotted as a function of time.
Results and discussion

Bacterial growth in the micofluidic chambers

First growth conditions and diffusion characteristics were determined inside the chambers. The goal was to reach a chemostatic environment to extend the growth of the bacterial culture to an extremely high density without being inhibited by nutrient depletion or waste accumulation. At 30°C with a constant flow of fresh LB medium in the channels, the generation time was determined using the fluorescence output from the cells (when grown with a constant level of exogenous autoinducer) to be about 55 minutes. The exponential rate of growth was between 0.0143 min\(^{-1}\) and 0.0125 min\(^{-1}\) (personal communication with Dr. Andre Levchenko) for E. coli strain JM109 carrying the different plasmids used in this study (Table 3.1) and expressing a fluorescent phenotype, until the chamber is densely filled with cells. This growth rate is close to the one observed in liquid batch culture at the same temperature (about 50 minutes when expressing fluorescence). Once the chamber space available is completely occupied by the cells, the rate of growth diminishes because the nutrient diffusion rate is limited and there is an internal pressure build up in the chambers. The pressure increase ultimately deforms the chambers and the slits to finally let the cells escape the chambers as they continue to grow. However, the chemostatic conditions in the chambers were maintained as the cells were growing at a consistent rate, until reaching extremely high density.

Table 3.1. Bacterial strain and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi(\Delta(lac-proAB)) F(\prime) [traD36 proAB(^{-}) lacI(^{0}) lacZ(\Delta M15)]</td>
<td>(85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E. coli Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGFI</td>
<td>Ap(^{r}); pBBR1-luxR-luxI-RBS-gfp(\text{mut3}^{*})-T(_{0})</td>
<td>This study</td>
</tr>
<tr>
<td>pJWP02</td>
<td>Km(^{r}); pBBR1-luxR-luxI-RBS-gfp(LAA)-T(_{0})</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA01</td>
<td>Ap(^{r}); pUC18Not-luxR-P(<em>{luxI})-RBSII-gfp(LVA)-T(</em>{0})-T(_{1})</td>
<td>This study</td>
</tr>
<tr>
<td>pLVA02</td>
<td>Ap(^{r}); pUC18Not-luxR-luxI-RBSII-gfp(LVA)-T(<em>{0})-T(</em>{1})</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Expression and detection of fluorescence**

The following work was done by Caroline Lobo at Dr. Levchenko’s laboratory with the strains that she was provided by me. To characterize the onset of induction of the quorum sensing system and the detection of fluorescence in the microfluidic system *E. coli* cells transformed with pLVA01 were grown in the chambers and then they were exposed to 10 nM of a racemic mixture 3-oxo-hexanoyl homoserine lactone in the medium flowing in the channels. The autoinducer dependent *lux* promoter fused to a *gfp* gene was used to determine the diffusion characteristics of autoinducer molecules in the chambers. Observations showed a homogenous and synchronous induction of fluorescence throughout the population with no significant noise. The diffusion of autoinducer molecules in the system is therefore believed to be significantly faster than cell division and expression of the reporter gene. To characterize the time response to the signal and the half life of the unstable variant of the green fluorescent protein, GFP(LVA), autoinducer was introduced first and removed from the flowing medium 5 hours after its introduction. The level of fluorescence in the chamber was followed. A sharp increase of detected fluorescence occurred with a delay of about 20 minutes following addition of autoinducer. Transcription and translation happen within a few minutes in *E. coli* but this delay corresponds to the relatively long maturation process of the GFP protein (between 20 and 30 minutes (11)). Then the slower exponential increase in fluorescence (0.013 min\(^{-1}\)) is attributed to cell growth when GFP(LVA) synthesis reached its steady state. The rate of degradation of GFP(LVA) was determined after removal of autoinducer from the flowing medium. After an intermediate state of 45 min where autoinducer was progressively washed from the chambers, degradation occurred at an exponential rate of 0.0178 min\(^{-1}\) (half-life of 39 min). This value is consistent with what was determined for GFP(LVA) in *E. coli* batch culture (0.018 min\(^{-1}\)) (3). The use of unstable GFP(LVA) in the microfluidic system was confirmed to be suitable to study transient transcription regulation. However the relatively long maturation time and half life stay a concern especially when the study of down-regulation related to biphasic regulation at high autoinducer level will be undertaken.
Observations of the quorum sensing regulation

Quorum sensing regulation in the chambers was observed when growing strains of *E. coli* transformed with vectors carrying the two essential genes for a functional quorum sensing response: *luxR* and *luxI*. Growth assays were done with *E. coli* transformed with either pLVA02 or pGFI, which contain the same *lux* gene fragment. In this protocol the cells produced their own signal and the response was revealed by either Gfpmut3* with pGFI or GFP(LVA), the unstable variant of Gfpmut3*, with pLVA02; both co-expressed with LuxI. The first experiments were done in chambers with a height of about 5 μm. Cell growing in those chambers had a degree of freedom in the z dimension permitting them to move out of the microscope focus. The cells therefore grew in multiple layers making discrimination of single cells impossible. However the fluorescence level emanating from out-of-focus cells could be recovered by the camera. As a result, the overall fluorescence characterized the population response in the chambers. Total fluorescence recovered as they grew in the chambers is presented in Figure 3.2. Quorum sensing system induction happened when the population reached between 20 and 50 members. Past the point of induction, visual observations did not reveal significant noise in the expression of fluorescence from cell to cell.

Analysis of the curves revealed that in both strains the starting exponential rates of increase in fluorescence (0.0293 min⁻¹ for pLVA02 and 0.0286 min⁻¹ for pGFI) are faster than the rate of growth determined previously (between 0.0143 min⁻¹ and 0.0125 min⁻¹, see “Bacterial growth in the microfluidic chambers”). It is proposed that this rate was a result of induction of the synthesis and accumulation of GFP in cells while the culture was growing. The maturation and degradation rate of Gfpmut3* and even GFP(LVA) are not significantly small compared to the bacterial division rate. The rates then progressively decrease to reach values closer to bacterial growth rates as the cells get saturated with GFP. Quantitative decoupling of those rates was not possible in the experiments done because rates of growth could not be determined. A fluorescence reporter of another color (probably a red fluorescent protein) expressed constitutively by the cells or a non toxic total cell stain will be introduced in the future to be able to follow the rate of growth independently from the quorum sensing response.
Figure 3.2. Evolution of total fluorescence followed through growth of *E. coli* carrying either pGFI (red) or pLVA02 (blue) in one microfluidic chamber. The strains were able to produce their own signal, therefore autotinduction of the fluorescent reporter happened when the population reached the threshold density. The exponential rates fitted to the experimental values are indicated next to the curves. Results represent one trial of an assay done in duplicate.
The affect of the instability of GFP(LVA) against its stable counterpart was also hard to characterize even though its appears on the Figure 3.2 and by visual examination of the pictures that the cells did express a weaker level of fluorescence per cell. Another point to consider is that induction happened with a very small quorum. This is a concern that needs to be addressed if further studies of the spatial organization of the community will be undertaken. Indeed, a delayed induction could provide a better model to investigate the spread of signaling throughout an already spatially established population. Sensitivity to small variations in cell density and down-regulation at high density were proposed to be critical in biofilm structure creation.

**Observations of the quorum sensing regulation in shallow chambers**

A different design of chambers was used to oblige the cells to grow in only one layer and keep them focus under the microscope. The chambers used were only about 1 µm in height hence limiting movement in the z direction. *E. coli* carrying plasmid pJWP02 coding for a functional quorum sensing system linked to an unstable fluorescent protein (GFP(LAA) which has the same half life as GFP(LVA) in *E. coli* (3)) was introduced into the microfluidic chambers. Autoinduction and expression of fluorescence happened when the quorum reached about 20 cells. Since it is impossible to correlate density in the chambers with optical density in batch culture, dynamics of the quorum sensing system cannot be compared with results obtained with the same *E. coli* strain in liquid culture. Early induction in the chambers is certainly due to the extremely confined space available, greatly reducing diffusion of autoinducer. Figure 3.3 is a plot of the total fluorescence recovered from the chamber over time. The first step on the curve happening after 100 minutes corresponds to the first appearance of fluorescence after induction of the quorum sensing response. Then the rate of fluorescence output stayed consistent at 0.0143 min⁻¹ for about 6 hours of growth. The transition to a slower rate of growth (0.0073 min⁻¹) coincided with the moment when cells completely filled the chamber. However, cells continued to divide and the pressure built up eventually deforming the chambers, allowing the cells to stack up in several layers and finally to escape through the slits. The single cell resolution was lost but the density reached was extremely high.
Figure 3.3. Evolution of total fluorescence followed through growth of *E. coli* carrying pJWP02 in one microfluidic chamber. The strain was able to produce their own signal, therefore autoinduction of the fluorescent reporter happened when the population reached the threshold density. The exponential rates fitted to the experimental values are indicated next to the curves. Results represent one trial of an assay done in duplicate.
In this situation the rates of fluorescence increase observed may reflect more accurately the actual bacterial growth rate. The values are very close to the rate of growth previously determined (between 0.0143 min\(^{-1}\) and 0.0125 min\(^{-1}\)). The combination of a limited diffusion of autoinducer in the chamber and the instability of GFP(LAA), allowed the steady state of fluorescence per cell to be reached faster than the rate of growth. In addition, pJWP02 is based on a pBBR1 replicon which is maintain in medium copy number in \(E.\ coli\) (4), that possibly induced a lower level of expression of the fluorescent reporter.

Observations of a series of pictures reveal the potential of the microfluidic system (Figure 3.4). The resolution obtained with this chamber design is fascinating as single cells are trapped in a defined space and can be visually isolated and be followed over time. Image analysis software tools are in development to achieve this type of analysis and distinguish the quorum sensing regulation dynamic for each cell. Figure 3.4 shows a sequence of images extracted from the time series. It shows the colony growing and progressively filling the chamber arranged in one layer (panel A, B and C). Then, after the chamber was filled, the pressure build-up forcing them to stack up in several layers (panel D, E and F). It can be noted that the population response presented some noise but no spatial disparities (e.g. cells at the edge versus center). The brighter areas between the slit openings on each sides of the chamber are likely to be a result of discrepancies in height presented by this particular chamber. Another intriguing characteristic was the occurrence of black spots through the colony. It is supposed that they were dead cells because through examination of image series, none of them appeared to divide and the space was not taken over by other cells despite the pressure build-up. Panels D, E and F in Figure 3.4 show the increasing proportion of black cells in the colony during the late phase of growth. The event of supposedly cell death was sudden as presented in Figure 3.5. The frames are taken with 5 minute intervals and the cells turned from bright to black between two frames. Considering the relatively long half life of GFP(LAA), the cells must have experienced a dramatic event like cell lysis. However, some cell structure remained as they continued to occupy a defined space. Although final conclusions cannot be made before a systematic analysis is performed, it seemed that those cells were brighter than the average right before their disappearance (as exposed in Figure 3.5).
Figure 3.4. Pictures extracted from the series illustrating growth and quorum sensing response of *E. coli* carrying pJWP02 in a shallow microfluidic chamber. The cells were able to produce their own signal and the response was reported by a fluorescent phenotype. The chamber corresponds to the one presented in Figure 3.1. The data plotted in Figure 3.3 were extracted from the original picture series. The pictures have been cropped from their original size. (A at 220 min, B at 535 min, C at 670 min, D at 945 min E at 1075 min and F at 1340 min).
Figure 3.5. Pictures illustrating disappearance of fluorescence in a colony of *E. coli* carrying pJWP02 grown in a microfluidic chamber. The four pictures constitute a sequence with 5 minutes intervals. They were extracted from the same picture series as pictures from Figure 3.4.
Conclusions

Observations done in the microfluidic device demonstrated the benefits offered in the prospect of studying bacterial signaling and multicellular organization. First, it achieved chemostatic conditions and permitted temporal control on the medium composition and temperature. The cells were protected from the perturbation engendered from the medium flow. Besides, the well-developed process of fabrication of the PDMS chips allows the development of new chip design with little constraints on the geometry. Desired conditions can be easily created for the experimental subject (for this study, our strain of E. coli). Therefore situations close to the natural environment can be reproduced. With this design, the dimensions of the chambers used were close to the size of the crypts within the V. fischeri host light organ (53, 54). The primary goal to bring the colony to extremely high density while sustaining a steady rate of growth was achieved. Even exceptionally shallow chambers offered good environmental conditions to allow interesting phenomena observations. The resolution and sensitivity offered by the fluorescent reporter were satisfying enough to suggest that a more thorough analysis at the single cell level will be possible in the future.

However in the current state of development, caveats remain to be addressed. First a reporter must be developed to quantify the rate of bacterial growth independently from the quorum sensing response. Secondly, the quorum sensing system autoinduction happened with a quorum of only a dozen cells. This situation excludes the possibility to further study the spread of the signal through an already structured community.

Finally, the quantitative data presented in this chapter are not sufficient to derive parameter constants or make definitive conclusions about the system; however the development of a mathematical model, presented in the next chapter, is an attempt to provide more insights in the dynamics of the quorum sensing system and define quantitatively kinetic parameters.
Chapter Four: Mathematical Modeling

Introduction

The project ultimately has the ambition to describe in quantitative terms the modalities of cell-to-cell communication in biofilm conditions. Dr. Andre Levchenko developed a cell automaton simulation to investigate the diffusion of the signal in a spatially organized bacterial population. On the other hand, individual response from the cells and synthesis of the signal are other critical components of the overall cell-communication picture.

The quorum sensing system is a highly dynamic molecular mechanism to regulate gene expression in a density dependent manner. However due to the nature of the system, it does not depend solely on population density. The diffusion rate of the signal in the environment or the physiological state of the cells, are also critical factors. Identifying the critical parameters for a set of conditions may be challenging. Understanding the system regulation becomes non-intuitive as it is combined with other regulatory systems. Therefore, a careful quantitative analysis needs to be undertaken. This necessity appears clear in the situation where several quorum sensing systems are interconnected in one organism (e.g. in the biofilm-forming bacterium *Pseudomonas aeruginosa*). The modeling efforts have started with the *V. fischeri* quorum sensing system because it presents the advantage to be the simplest system known to date. That allows an extensive experimental and quantitative analysis of the basics of a system shared by many organisms.

This chapter is a first stage of the development of a mathematical description of the molecular mechanisms of the quorum sensing system in one cell. The quorum sensing system has already drawn attention of modelers for its dynamic behavior (9, 12, 17, 36, 81, 82). However, none of these models were supported by a thorough experimental effort to carefully define the kinetic constants of the system. The work undertaken here was partially based on the model developed by Cox et al. (12) with a more systematic description of the molecular mechanism and its dynamics. An initial attempt to compare experimental data to model simulations is also presented.
The biochemical model

The biochemical model for the quorum sensing system was inspired from the literature (see Chapter 1: Literature review). Regulation of the whole system is rather simple and occurs primarily at the level of gene transcription. The rate of transcription of both $luxR$ and the $lux$ operon is controlled by the binding of the LuxR-AI dimer complex to the $lux$ box, situated in the intergenic promoter region of $luxR$ and $luxI$, or on the $luxD$ box. When the two boxes are occupied by the dimer, a DNA loop may form to create a contact between the dimers bound on the DNA. Therefore the transcription levels of the two transcriptional units are dependent on five possible combinations. Combination 1 (C1) represents the state where both boxes are free; C2: only the $luxD$ box is occupied; C3: only the $lux$ box is occupied; C4: both boxes are occupied; C5: a DNA loop forms between the two boxes when they are both occupied.

**Figure 4.1.** Wiring diagram of the quorum sensing system. Solid arrows represent the biochemical reactions (transcription, translation, degradation (*), diffusion or binding); dashed arrows represent influences (catalysis or transcription activation or repression). A biochemical kinetic constant is associated with each arrow. Reversible reactions are indicated by double-end arrows. This diagram was inspired from (12).
The rate of transcription is at the basal level when the system is in the states C1 and C2. Transcription is induced in the states C3 and C4 and repressed by DNA looping in the state C5.

**The mathematical model**

The mathematical model uses ordinary differential equations to translate the biochemical network in quantitative terms. The equations were written based on the wiring diagram (Figure 4.1) using the laws of mass action or Michaelis-Menten kinetics. All species (proteins or molecules) are treated as variables while kinetic constants were assigned to the biochemical reactions driving the system (arrows). The kinetic parameters were either determined from the literature or estimated in such a manner that they are in a physiologically relevant range and the model outputs fit the experimental data. However the parameter space that would satisfy the constraints imposed by the experimental data considered in this chapter is very large. Further experimental analyses are needed to characterize precise values for these biochemical constants. The values used for the kinetic constants are summarized in Table 4.1. The model is mainly used in this chapter to investigate the principal characteristics of the quorum sensing system, and to provide a working template for further development. The effect of the presence of the luxD box and repression of transcription by DNA looping is theoretically addressed.

The dynamics of the system are dependent on the rate of the successive biochemical reactions. However they do not all occur at the same rate. It is the limiting steps (slower reactions) that have the greater affect on the system and define its behavior. In the quorum sensing system, the coupled transcription and translation and autoinducer synthesis are the slower reactions and were described using ordinary differential equations. Binding of the LuxR-AI dimer complex on the DNA is significantly faster. Therefore its equations are written for the steady-state solution.
<table>
<thead>
<tr>
<th>Reactions</th>
<th>Parameters (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA → Protein</td>
<td>(k_{\text{m}} = 1.4)</td>
</tr>
<tr>
<td>mRNA → *</td>
<td>(\gamma_{\text{m}} = 0.2)</td>
</tr>
<tr>
<td>luxR → LuxR (basal level)</td>
<td>(k_{\text{basalR}} = 0.05)</td>
</tr>
<tr>
<td>luxR → LuxR (induced level)</td>
<td>(k_{\text{maxR}} = 0.5)</td>
</tr>
<tr>
<td>LuxR → *</td>
<td>(\gamma_{\text{pR}} = 0.15)</td>
</tr>
<tr>
<td>2 LuxR + 2 AI → (LuxRAI)(_2)</td>
<td>(k_{\text{f1}} = 0.1)</td>
</tr>
<tr>
<td>(LuxRAI)(_2) → 2 LuxR + 2 AI</td>
<td>(k_{\text{r1}} = 10)</td>
</tr>
<tr>
<td>(LuxRAI)(_2) → *</td>
<td>(\gamma_{\text{pRAI}} = 0.075)</td>
</tr>
<tr>
<td>(C_1 + (\text{LuxRAI})_2 \rightarrow C_3)</td>
<td>(k_{\text{f3}} = 0.5)</td>
</tr>
<tr>
<td>(C_1 + (\text{LuxRAI})_2 \rightarrow C_2)</td>
<td>(k_{\text{f4}} = 0.05)</td>
</tr>
<tr>
<td>(C_2 \rightarrow C_1 + (\text{LuxRAI})_2)</td>
<td>(k_{\text{r4}} = 2.5)</td>
</tr>
<tr>
<td>(C_3 \rightarrow C_1 + (\text{LuxRAI})_2)</td>
<td>(k_{\text{r3}} = 2.5)</td>
</tr>
<tr>
<td>(C_3 + (\text{LuxRAI})_2 \rightarrow C_4)</td>
<td>(k_{\text{f4}} = 0.05)</td>
</tr>
<tr>
<td>(C_4 \rightarrow C_3 + (\text{LuxRAI})_2)</td>
<td>(k_{\text{r4}} = 2.5)</td>
</tr>
<tr>
<td>(C_4 \rightarrow C_5)</td>
<td>(k_{\text{floop}} = 10)</td>
</tr>
<tr>
<td>(C_5 \rightarrow C_4)</td>
<td>(k_{\text{floop}} = 1)</td>
</tr>
<tr>
<td>luxI → LuxI (basal level)</td>
<td>(k_{\text{basalI}} = 0.025)</td>
</tr>
<tr>
<td>luxI → LuxI (induced level)</td>
<td>(k_{\text{maxI}} = 2.5)</td>
</tr>
<tr>
<td>LuxI → *</td>
<td>(\gamma_{\text{pI}} = 0.2)</td>
</tr>
<tr>
<td>LuxI → LuxI + AI</td>
<td>(k_{\text{ai}} = 0.2)</td>
</tr>
<tr>
<td>AI → AI(_{\text{ext}})</td>
<td>(k_{\text{out}} = 36)</td>
</tr>
<tr>
<td>AI(_{\text{ext}}) → *</td>
<td>(\gamma_{\text{ai}} = 12)</td>
</tr>
<tr>
<td>proGFP → *</td>
<td>(\gamma_{\text{proGFP}} = 0.27)</td>
</tr>
<tr>
<td>proGFP → GFP</td>
<td>(k_{\text{mat}} = 0.04)</td>
</tr>
<tr>
<td>GFP → *</td>
<td>(\gamma_{\text{GFP}} = 0.018)</td>
</tr>
<tr>
<td>Rate of bacterial growth</td>
<td>(r = 0.0138)</td>
</tr>
</tbody>
</table>
The following set of equations describes the probability for each box to be occupied by (LuxRAI)₂.

\[
\begin{align*}
C_1 + C_2 + C_3 + C_4 + C_5 &= 1 \\
C_1 \cdot kf_4 \cdot [(LuxRAI)_2] &= C_2 \cdot kr_4 \\
C_1 \cdot kf_3 \cdot [(LuxRAI)_2] &= C_3 \cdot kr_5 \\
C_2 \cdot kf_3 \cdot [(LuxRAI)_2] &= C_4 \cdot kr_5 \\
C_3 \cdot kf_4 \cdot [(LuxRAI)_2] &= C_4 \cdot kr_4 \\
C_4 \cdot kf_{loop} = C_5 \cdot kr_{loop}
\end{align*}
\] {1}

Analytical solutions:

\[
C_1 = \frac{(kr_\ell \cdot kr_\ell \cdot kr_{loop})}{(kr_\ell \cdot kr_\ell \cdot kr_{loop}) + (kr_{loop} \cdot (kf_\ell \cdot kr_\ell + kf_\ell \cdot kr_\ell) \cdot [(LuxRAI)_2]) + (kf_\ell \cdot kf_\ell \cdot (kf_{loop} + kr_{loop}) \cdot [(LuxRAI)_2])}
\]

\[
C_2 = \frac{(kr_\ell \cdot kr_\ell \cdot kr_{loop})}{(kr_\ell \cdot kr_\ell \cdot kr_{loop}) + (kr_{loop} \cdot (kf_\ell \cdot kr_\ell + kf_\ell \cdot kr_\ell) \cdot [(LuxRAI)_2]) + (kf_\ell \cdot kf_\ell \cdot (kf_{loop} + kr_{loop}) \cdot [(LuxRAI)_2])}
\]

\[
C_3 = \frac{(kf_\ell \cdot kr_\ell \cdot kr_{loop})}{(kr_\ell \cdot kr_\ell \cdot kr_{loop}) + (kr_{loop} \cdot (kf_\ell \cdot kr_\ell + kf_\ell \cdot kr_\ell) \cdot [(LuxRAI)_2]) + (kf_\ell \cdot kf_\ell \cdot (kf_{loop} + kr_{loop}) \cdot [(LuxRAI)_2])}
\]

\[
C_4 = \frac{(kf_\ell \cdot kf_\ell \cdot kr_{loop})}{(kr_\ell \cdot kr_\ell \cdot kr_{loop}) + (kr_{loop} \cdot (kf_\ell \cdot kr_\ell + kf_\ell \cdot kr_\ell) \cdot [(LuxRAI)_2]) + (kf_\ell \cdot kf_\ell \cdot (kf_{loop} + kr_{loop}) \cdot [(LuxRAI)_2])}
\]

\[
C_5 = \frac{(kf_\ell \cdot kf_\ell \cdot kr_{loop})}{(kr_\ell \cdot kr_\ell \cdot kr_{loop}) + (kr_{loop} \cdot (kf_\ell \cdot kr_\ell + kf_\ell \cdot kr_\ell) \cdot [(LuxRAI)_2]) + (kf_\ell \cdot kf_\ell \cdot (kf_{loop} + kr_{loop}) \cdot [(LuxRAI)_2])}
\]

Figure 4.2 illustrates the resulting probability for each state of the system according to the concentration of (LuxR-AI)₂. When the concentration of the complex dimer increases (induced by the increase in autoinducer intracellular concentration), the lux box is first occupied to induce transcription of both transcriptional units. Then, as more LuxR-AI complexes accumulate into the cell, the luxD box is occupied and a DNA-loop forms to repress transcription of luxR. The effect of the luxD box can be dramatic according to the set of parameters used. Figure 4.2 shows that the system shifts from the induced state (C3+C4, green curve) to the repressed state (C5, red curve) as the concentration of the LuxR-AI complex increases.
Figure 4.2. Probability to find the quorum sensing output in one of three transcriptional regulation states as the concentration of \((\text{LuxR-AI})_2\) increases (arbitrary units). (Blue: Transcription at the basal level (C1+C2), Green: Transcription at the induced level (C3+C4), Red: Transcription repressed by DNA looping (C5)). See text for details.

The following set of ordinary differential equations describes the variations in concentration of the different species of the system:

\[
\begin{align*}
\frac{d[	ext{LuxR}]}{dt} &= \frac{k_{ul}}{\gamma_m} \cdot (k_{basalR} \cdot (C_1 + C_2) + k_{maxR} \cdot (C_3 + C_4)) - 2 \cdot k_{f1} \cdot ([\text{LuxR}] \cdot [\text{AI}])^2 \\
&\quad + 2 \cdot k_{r1} \cdot ([\text{LuxRAI}]_2) - \gamma_{pR} \cdot [\text{LuxR}] \\
\frac{d[\text{LuxI}]}{dt} &= \frac{k_{ul}}{\gamma_m} \cdot (k_{basalI} \cdot (C_1 + C_2) + k_{maxI} \cdot (C_3 + C_4)) - \gamma_{pl} \cdot [\text{LuxI}] \\
\frac{d[\text{AI}]}{dt} &= k_{ai} \cdot [\text{LuxI}] - 2 \cdot k_{f1} \cdot ([\text{LuxR}] \cdot [\text{AI}])^2 + 2 \cdot k_{r1} \cdot ([\text{LuxRAI}]_2) \\
&\quad - k_{out} \cdot ([\text{AI}] - [\text{AI}_{ext}]) \\n\frac{d[\text{AI}_{ext}]}{dt} &= e^{l_t} \cdot (k_{out} \cdot ([\text{AI}] - [\text{AI}_{ext}])) - \gamma_{ai} \cdot [\text{AI}_{ext}] \\
\frac{d([\text{LuxRAI}]_2)}{dt} &= k_{f1} \cdot ([\text{LuxR}] \cdot [\text{AI}])^2 - k_{r1} \cdot ([\text{LuxRAI}]_2) - \gamma_{pRAI} \cdot ([\text{LuxRAI}]_2) 
\end{align*}
\]

The extracellular concentration of autoinducer is dependent on the number of cells in the population. Population growth is considered here as a simple exponential growth, for the
reason that later evaluations of the model will be done against experimental data from a culture that was in its exponential phase of growth.

The basal transcription rates of luxR or luxI are associated with the states C1 and C2, when the lux box is empty. When the LuxR-AI complex is bound to the lux box (C3 and C4) luxR and luxI are transcribed at the maximum rates. Notice that the state C5 is not associated to a transcription rate of luxR or luxI in \{2\}. C5 is considered as a fully repressed state. Down-regulation of transcription from the lux operon had not been demonstrated yet, but experimental evidence indicated a repression of the transcription of the lux operon at high concentrations of autoinducer (Figure 2.5). Nevertheless, further analysis is needed to confirm this hypothesis.

The model simulations were done in the Mathematica® 4.2 environment (Wolfram Research, Inc., Champaign, Il).

**The effect of the luxD box on transcription regulation**

Next, the effect of the luxD box on system regulation is considered. When the luxD box is not present, the states C2, C4 and C5 disappear; therefore the equations for LuxR and LuxI are rewritten from \{2\} as:

\[
C_1 = \frac{kr_3}{kr_3 + (kf_3 \cdot [(\text{LuxRAI})]_2)}
\]

\[
C_3 = \frac{kf_3 \cdot [(\text{LuxRAI})]_2}{kr_3 + (kf_3 \cdot [(\text{LuxRAI})]_2)}
\]

\[
\frac{d[\text{LuxR}]}{dt} = \frac{k_{dl}}{\gamma_m} \cdot \left( k_{basal R} \cdot \left( C_1 \right) + k_{max R} \cdot \left( C_3 \right) \right) - 2 \cdot k_{f_1} \cdot \left( [\text{LuxR}] \cdot [\text{AI}] \right)^2 + 2 \cdot k_{f_1} \cdot [(\text{LuxRAI})]_2 - \gamma_p \cdot [\text{LuxR}]
\]

\[
\frac{d[\text{LuxI}]}{dt} = \frac{k_{dl}}{\gamma_m} \cdot \left( k_{basal I} \cdot \left( C_1 \right) + k_{max I} \cdot \left( C_3 \right) \right) - \gamma_p \cdot [\text{LuxI}]
\]

Then the solutions for the steady states of [LuxR] and [LuxI] are determined to express their respective concentrations inside the cell in term of the intracellular concentration of autoinducer (Figure 4.3). The model predicts that the presence of the luxD box induces a biphasic expression of both transcriptional units as the concentration of autoinducer increases (dashed lines). On the other hand, the absence of the luxD box allows a higher expression of the proteins in a monotonic fashion (solid lines).
Dependence of the concentrations of LuxR (A) and LuxI (B) on the concentration of intracellular autoinducer. The curves are the results of simulations when the \textit{luxD} box is present (dashed lines) or when it is absent (solid lines) (arbitrary units).

\textbf{Induction of quorum sensing}

Further simulations presented in this chapter were done with the \textit{luxD} box absent since most available experimental data at this point are from a quorum sensing system missing the \textit{luxD} gene. The set of equations used is therefore \{3\}.

To better understand the transition of the quorum sensing system from the non-induced state to the induced state, a phase portrait for the dynamical system is plotted in Figure 4.4. It represent the nullclines for [LuxR] and [AI] at different concentration of the extra-cellular autoinducer ([AI\textsubscript{ext}]=1.1, 1.55 or 2.0, arbitrary units). The nullclines are obtained when the differential equations of the system are set individually to zero and each equation is solved as a function of the other variable (here, d[LuxR]/dt=0 as a function of [AI] or d[AI]/dt=0 as a function of [LuxR]). Biologically it corresponds to combinations of [LuxR] and [AI] where the rates of production of species are exactly balanced by the rates of removal. At the intersection of the nullclines, neither variable is changing in time; therefore the dynamical system has a steady-state solution. These steady-states can be stable (●) or unstable (○). A stable steady-state means that if the system is perturbed to some extent (noise in gene expression or protein levels, small variations in the autoinducer concentration), the system will come back to its previous steady-state. For an unstable steady-state, small perturbations will drive the system away from the steady-state. Because perturbations always occur in biological system, an unstable steady-state cannot be sustained. It often represents a switch between two stable steady-states.
The phase portrait is plotted from these two equations:

\[
\frac{d[LuxR]}{dt} = \frac{k_{dl}}{\gamma_m} \cdot \left( k_{basalR} \cdot (C_1) + k_{maxR} \cdot (C_3) \right) - \gamma_pR \cdot [LuxR] \\
\frac{d[AI]}{dt} = \frac{k_{dl}}{\gamma_m} \cdot \frac{k_{ai}}{\gamma_pI} \cdot \left( k_{basalI} \cdot (C_1) + k_{maxI} \cdot (C_3) \right) - k_{out} \cdot ([AI] - [AIext])
\]

Figure 4.4 shows that at a low extra-cellular autoinducer concentration ([AI\_ext]=1.1) the cell is maintained in a non-induced state (low expression of LuxR and low synthesis of AI). The cell cannot escape this state by itself. At very high extra-cellular autoinducer concentration ([AI\_ext]=2.0), the quorum sensing system is induced. The production of LuxR and autoinducer is maintained high. Interestingly there is region of bistability around the threshold concentration of autoinducer required to induce the system ([AI\_ext]=1.55).

**Figure 4.4.** Phase portrait of the dynamics of the concentration of LuxR and AI as a function of the extracellular autoinducer. The black line is [LuxR]-nullcline (where d[LuxR]/dt=0) and the vertical color lines are [AI]-nullclines (where d[AI]/dt=0) for indicated [AI\_ext] (arbitrary units). The system steady-state solutions are represented at the intersections of the nullclines ([●] when stable and (o) when unstable).

As the extra-cellular concentration of autoinducer increases, the system reaches the lower saddle-node bifurcation (at the lower folding point of the [LuxR] curve where the low stable steady-state coincides with the unstable steady-state and both disappear) and the
concentration of LuxR is lifted to its induced state concentration. In the other direction, when the cells start from an induced state and the extra-cellular concentration of autoinducer decreases, the system is switched to its non-induced state when it reaches the upper saddle-node bifurcation (upper folding point of the [LuxR] curve).

This characteristic means that the quorum sensing system needs a higher extra-cellular autoinducer concentration to induce than it needs to be able to stay induced. That provides robustness to the system. However, the saddle-node bifurcation depends on the parameter values of the model. Experimental analyses are needed to confirm that robustness is observed in the *V. fischeri* quorum sensing system. A more careful bifurcation analysis is needed to identify the critical parameters that would affect the robustness in the system.

**Simulations from the model**

To initiate the effort to fit the mathematical model to experimental data, equations describing the transcription, translation and maturation of GFP were written. As described earlier (Chapter 2 and 3), light production of the quorum sensing system was replaced by a fluorescent reporter. It is necessary to take in account in the model the relatively long time of maturation and half-life of GFP (different parameters can be used according to the variant of GFP used in the experimental analysis) because they induce a lag in the time of the response of the reporter (41).

The following set of equations describe the coupled transcription and translation of the non-mature GFP (termed “proGFP”), and its maturation process to a fluorescent protein.

\[
\frac{d[\text{proGFP}]}{dt} = \frac{k_{dl}}{\gamma_m} \cdot \left( k_{basal} \cdot (C_1) + k_{max} \cdot (C_3) \right) - \gamma_{proFP} \cdot [\text{proGFP}] \\
\frac{d[\text{GFP}]}{dt} = k_{mat} \cdot [\text{proGFP}] - \gamma_{GFP} \cdot [\text{GFP}] \tag{5}
\]

Equations \{1\}, \{2\}, \{3\} and \{5\} were combined to create a full model representing the experimental system presented in Chapter 3 (*E. coli* JM109 transformed with pJWP02 and grown in a shallow microfluidic chamber (Figure 3.3)). The luxD box is absent from this system, therefore down-regulation was not considered. Figure 4.5 represents the results of the simulation of population growth and induction of the quorum sensing
system when the threshold density is reached. The rate increase of cell number was set to match observations done in the microfluidic chamber.

Figure 4.6 represents the plot of the fluorescence output per cell from the experiment along with the model simulation. The monotonic expression of the fluorescent reporter in the experimental system corroborates the assumptions used for model.
Figure 4.5. Simulations from the mathematical model. **A.** Population growth. The rate of growth was set to match the one observed in the microfluidic chamber experiment. **B.** Concentration of autoinducer per cell. The concentration increases exponentially because the whole cell population contributes to AI synthesis. However, the concentration eventually reaches a steady-state. **C.** Concentration of free LuxR (violet), total LuxR (blue) and \((\text{LuxRAI})_2\) per cell. **D.** Concentration of LuxI (blue) and GFP (green) per cell. The GFP concentration increases more slowly because of its long maturation time.
Figure 4.6. Evolution of the concentration of GFP(LVA) per cell over time. Both the experimental data (black dots) and the simulation result (blue solid line) are plotted. When the quorum sensing system was induced (after about 70 min) the concentration of the fluorescent reporter sharply increased. The simulation does not account for stochastic variations of the level of expression. The monotonic expression of the fluorescent reporter in the experimental system corroborates the assumptions used for the model. The apparent oscillations of the experimental data are an artifact from microscopy.
Conclusions

The development of a mathematical model for quorum sensing in *Vibrio fischeri* is still at an early stage. Most of the parameters were roughly estimated to fit the experimental data presented and extended levels of regulation of the system were disregarded. Nonetheless, the model successfully described the qualitative behavior of the quorum sensing system. It is characterized by two states of activity under a cell-density dependent control. A positive feedback loop creates a rapid switch between the two states and provides robustness to the dynamical system. The monotonic behavior predicted by the absence of negative feedback on the system was corroborated by experimental results. Furthermore, a mechanism to explain the biphasic behavior observed in certain experimental conditions was proposed. The *luxD* box appears to be a good candidate to provide control on the system at high autoinducer concentrations. However, the hypothesis of DNA looping theory remains to be demonstrated experimentally.

This model is meant to be a working template for further theoretical and experimental analyses. The system presented is rather simple and can be understood intuitively. Before assembling the pieces of a more complex regulatory network, such as cell-communication in biofilm formation, each piece has to be carefully defined. Then the pieces will fit together to give a compete picture of the complex behaviors that drive quorum sensing in living bacteria.
Chapter Five: Concluding Remarks

This project contained many original approaches and ideas. It included the development of innovative tools like the microfluidic chambers and utilized synergetic interactions between mathematical modeling and experimental analysis. This multifaceted approach to a broad and complex problem required substantial preliminary works to identify and develop the tools to be used. Consequently, the work presented here represents only the early stages of a long-term research strategy. It is meant to be the foundation for further developments in the plan of bringing both the experimental and theoretical tools together to undertake an extensive analysis of quorum sensing systems.

The creation of several genetic constructs using an unstable fluorescent reporter was motivated by the development of the microfluidic systems. Indeed, observations in the microfluidic chambers revealed the great potentials this system offers. The ability to observe the behavior of a bacterial community at the single-cell level in a favorable environment is an important component of our studies. Nevertheless, some adjustments remain to be made in both the genetic constructs and the microfluidic systems before reaching the desired level of performance.

The proposed mathematical model is also at an early stage of its development. It can account for the basic properties of the quorum sensing system, but it was not tested against a variety of conditions. Further comparisons between the mathematical simulations and experimental data are necessary to validate the model.

Among the specific goals that can be pursued next are system analyses, the definition of biochemical constants, such as the half-lives of the proteins, the signal diffusion rate and the rates of transcriptions are likely to be critical. The effect of the luxD box, which was tentatively assayed in this study, will require additional analysis. These individual characteristics will define the behavior of the quorum sensing response in each bacterial cell and ultimately the response of the community.

Bacterial communication has reached a high level of sophistication and represents a challenge in pursuing a complete understanding of bacterial behavior. Once viewed as self-sufficient organisms, bacteria have revealed many surprises in their ability to communicate across a population.
Chapter Six: References


Appendix

Figure 2.3. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in *V. fischeri*. Assays done with *Vibrio fischeri* strain MJ211 cell culture at an OD$_{600}$ of 0.5. Results represent one trial of an assay done in duplicate. (RLU/OD: relative light units per optical density unit).

Figure 2.4. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in *E. coli* pHV200I-. Assays done with *E. coli* strain VJS533 transformed with pHV200I- cell culture at an OD$_{600}$ of 0.5. Results represent an assay done in duplicate. The arrow bars are representative of the range for the values measured in triplicate. (RLU/OD: relative light units per optical density unit).
Figure 2.5. Biphasic response of the lux operon transcription to increasing amounts of autoinducer in *E. coli* pSC300 pJR551. Assays were done with *E. coli* strain JM109 (pJR551 pSC300) at an OD_{600} of 0.5. (A) Luminescence assay on 200 µl culture samples. (B) Luciferase assays on cell extract. (C) β-galactosidase assays on cell extract. The graphs are representative of experiments done in duplicate. Error bars represent the range of readings done in triplicate. (RLU/OD: relative light units per optical density unit).
Figure 2.6. Monotonic response of lux operon transcription to an increasing amount of autoinducer. Assays done on E. coli strain JM109 transformed with pJBA88 (● blue) or pLVA01 (▲ red) cell culture at an OD$_{600}$ of 0.8. Results represent one trial of an assay done in duplicate. Error bars represent the range of readings done in triplicate. (RFU/OD: relative fluorescence units per optical density unit).
Figure 2.7. Autoinduction of the lux operon transcription during bacterial growth with a β-galactosidase reporter. β-galactosidase assays done on E. coli strain JM109 transformed with pLUZ02 (▲ red), pLUZ03 (♦ blue), pLUZ04 (♦ violet) or pLUZ05(• green) cell culture. Results represent one trial of an assay done in triplicate. (RLU/OD: relative light units per optical density unit).
Figure 2.8. Autoinduction of the lux operon transcription during bacterial growth with a fluorescent reporter. Fluorescence measurements done on E. coli strain JM109 transformed with pGFI (● green), pJWP02 (■ yellow), pLVA12 (▲ orange) or pLVA02 (♦ blue) cell culture. Results represent one trial of an assay done in duplicate. (RFU/OD: relative fluorescence units per optical density unit).
Figure 2.9. Effect of the luxD box on the autoinduction of the lux operon transcription during bacterial growth. Fluorescence measurements done on E. coli strain JM109 transformed with pLVA03 (▲ blue) or pLVA04 (● red) cell culture. Results represent one trial of an assay done in duplicate. (RFU/OD: relative fluorescence units per optical density unit).
Figure 2.10. Effects of the introduction of different LuxR variants on the quorum sensing system response. Assays done with *E. coli* strain JM109 transformed with pJR551 and pSC300 (♦ blue) or its derivatives (alanine substitution at position 206 (● red), 213 (▲ orange), 216 (■ yellow) or 229 (ж green). The cultures were at an OD$_{600}$ about 0.8. Results represent one trial of experiments done in duplicate. (RLU/OD: relative light units per optical density unit).

Figure 2.11. Effects of the introduction of different LuxR variants on the quorum sensing system response after exposure to exogenous 3-oxo-hexanoyl-DL-homoserine lactone (AI). Assays done with *E. coli* strain JM109 transformed with the plasmids indicated on the graph. Results represent one trial of experiments done in duplicate. (RFU/OD: relative fluorescence units per optical density unit).
Figure 3.2. Evolution of total fluorescence followed through growth of *E. coli* carrying either pGFI (red) or pLVA02 (blue) in one microfluidic chamber. The strains were able to produce their own signal, therefore autinduction of the fluorescent reporter happened when the population reached the threshold density. The exponential rates fitted to the experimental values are indicated next to the curves. Results represent one trial of an assay done in duplicate.
Figure 3.3. Evolution of total fluorescence followed through growth of E. coli carrying pJWP02 in one microfluidic chamber. The strain was able to produce their own signal, therefore autoinduction of the fluorescent reporter happened when the population reached the threshold density. The exponential rates fitted to the experimental values are indicated next to the curves. Results represent one trial of an assay done in triplicate.
Curriculum Vitae

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EDUCATION

Master of Science, Microbiology, July 2004
Virginia Polytechnic Institute and State University, Blacksburg, Virginia

Bachelor of Science, Biology, Microbiology Concentration May 2002
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PROFESSIONAL EXPERIENCE

Graduate Research Assistant May 2003-Present
Department of Biology, Virginia Tech, Blacksburg, VA.
- Standard molecular biology techniques
- Physiological assays on bacterial cultures
- Luminescence and luciferase assays
- Mentored an undergraduate student
- Participated to departmental meeting and journal club
- Utilization of microfluidic analytical tools
- Fluorescence microscopy
- Development of an ODE based mathematical model

Graduate Teaching Assistant August 2002-May 2003
Department of Biology, Virginia Tech, Blacksburg, VA.
- Taught multiple sections of General Microbiology Laboratory
- Assisted in teaching a senior level Microbial Genetics and Physiology Laboratory
PRESENTATIONS

Development of a Quantitative Model for Quorum Sensing in *Vibrio fischeri*
July 2004, Cell-cell Communication in Bacteria ASM Meeting, Banff, Alberta, Canada.

Development of a Quantitative Model for Quorum Sensing in *Vibrio fischeri* (poster)
July 2004, Microbial Stress Response Gordon Research Conference, Mount Holyoke College, South Hadley, MA.

Systems Biology: a New Dimension in Biological Sciences
November 2003, Microbiology Departmental Seminar, Virginia Tech, Blacksburg, VA.

Experimental Analysis Necessary for the Development of a Computational Model for Quorum Sensing in *Vibrio fischeri*
October 2003, American Society for Microbiology Virginia Branch Local Meeting, University of Virginia, Charlottesville, VA.

Quorum Sensing in the *lux* Operon of *Vibrio fischeri* Shows a Biphasic Response to Increasing Amounts of Autoinducer (poster)
May 2003, American Society for Microbiology General Meeting, Washington, DC.

PUBLICATIONS


GRANTS

2004 - American Society for Microbiology Student Travel Grant Award for the 2nd ASM Conference on Cell-Cell Communication in Bacteria

2003 - American Society for Microbiology Student Travel Grant Award for the 103rd General Meeting

2003 - Research Development Project Grant from the Graduate Student Assembly

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

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