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Modeling Quorum Sensing in Sinorhizobium Meliloti

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Abstract: For bacteria, it is important for survival to sense their population density or quorum. We present a minimal mathematical model for quorum sensing in the nitrogen-fixing bacterium Sinorhizobium meliloti based on a system of differential equations, which was set up using data from microbiological experiments. The quorum sensing system depends upon the players ExpR, SinR, SinI and AHL, the signaling molecule within the system. The quorum sensing system undergoes three stages. At low cell density, all produced AHL molecules quickly leave the cells. In the medium, the concentration of AHLs increases only slowly. If the number of cells grows, the concentration of AHLs in the medium increases and AHLs can be transported back into the cell where they regulate their own production, i.e. they are responsible for a positive feedback loop. Finally, if cells are even more abundant, production of AHLs is switched off. Although our mathematical model is only qualitative it can correctly predict several empirical observations from wild-type and mutant strains.

AMS Subject Classification: 92C42 (Primary), 92C45, 92B05 (Secondary).

Keywords and Phrases: Cell-cell communication, Signaling, Ordinary differential equation, Multiscale system.

Introduction

Bacteria are one of the most prolific life forms known to man, successfully inhabiting a huge variety of habitats. A major contribution to their success is the ability to coordinate their behavior in response to the population density of the community, referred to as quorum sensing (QS). Numerous QS systems have been discovered throughout the bacterial world. These systems have been shown to enable cooperative responses that increase rates of survival, for example, through improved defense against competitors or hosts or through greater access to nutrients. Some well characterized examples are the pathogens *Agrobacterium*, *Pseudomonas*, *Erwinia*, and *Burkholderia*, where QS plays an important role in virulence via the regulation of processes such as plasmid conjugation, aggregation, exopolysaccharide production and exo-protease production. Other examples include the symbionts *Vibrio fischeri* and several rhizobial species *Mesorhizobium loti*, *Rhizobium leguminisarum* and *Sinorhizobium meliloti*, where QS is also found to either improve or to be essential for symbiosis. Yet other examples of QS regulated processes, that are beneficial for survival in the free living state of various bacteria are biofilm formation, competence, swarming motility, antibiotic production and spore formation (Nasser and Reverchon, 2007; Williams *et al.*, 2007; Antunes and Ferreira, 2009).

Previously, mathematical models have been used to study QS. Simple approaches distinguish between two types of cells, depending on their contribution to autoinducer (AI) production, without taking the molecular mechanisms of this contribution into account (Ward *et al.*, 2001). Several models consider the molecular mechanisms within a single cell, leading to a bi-stable behavior depending on a high or low AI concentration (Dockery and Keener, 2001; Müller *et al.*, 2006; Williams *et al.*, 2008; Kuttler and Hense, 2010) or the

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density of cells within the medium (Dockery and Keener, 2001). Moreover, in *V. fischeri*, it is known that the regulator LuxR forms a complex with the autoinducer AHL and then forms a dimer before it binds to the promoter region of the regulator, an effect taken into account in Müller *et al*., (2006) and Williams *et al*., (2008). All abovementioned modeling papers only explain the positive feedback which is responsible for distinguishing the basic state and cells which sense their quorum. In *S. meliloti*, we have to introduce a negative feedback which is responsible for switching off production of AHLs once the population has grown large enough. In addition, we take a practical approach and treat a spatial dimension in a mean-field way, i.e., we model a single cell and AI molecules in the surrounding medium. This is in contrast to using partial differential equations for transport of AIs, which are hard to fit to empirical data (Müller *et al*., 2006).

S. meliloti, a model organism for the study of rhizobia-legume symbiosis, contains a QS system called the Sin system. This system has been shown to significantly enhance symbiotic potential (Marketon *et al*., 2003; Patankar and González, 2009) and controls a variety of cellular processes such as cell division, cell motility, transcription, translation, metabolism and transport (Gao *et al*., 2005; Hoang *et al*., 2004, 2008). Most of the studies have focused on the activating role that the Sin system has on the production of symbiotically essential exopolysaccharides (Gurich and González, 2009; McIntosh *et al*., 2008; Marketon *et al*., 2003; Mueller and González, 2011; Pellock *et al*., 2002). The Sin system is based on several long-chain N-acyl homoserine lactones (AHLs) as signal molecules or autoinducers. The enzyme catalyzing the synthesis of these AHLs is SinI. *sinI* expression is regulated by SinR and ExpR (McIntosh *et al*., 2008; Pellock *et al*., 2002). SinR is essential for the expression of *sinI*, most likely by binding to the intergenic region between the two genes. However, its activity is independent of the Sin system AHLs. The AHL receptor is ExpR, encoded distant from *sinR* and *sinI*. In addition to regulating *sinI*, ExpR also controls genes in the production of symbiotically important exopolysaccharides and a variety of cellular processes such as cell division, transcription, translation, metabolism and transport (Hoang *et al*., 2004).

QS poses several conceptual questions: (i) It has been argued that bacteria not only sense their quorum, but the diffusion coefficient of their environment, which differs between liquid or a biofilm (Hense *et al*., 2007). These two assumptions have different consequences for the interpretation of QS. (ii) In many bacteria, there are multiple QS systems with inter-connected regulation. Hence, mathematical modeling of QS always suffers from studying open subsystems and only a delineation of the molecular basis of all QS systems within a bacterium can help to validate the modeling approach. In order to study such issues, an extensible quantitative model is needed.

In this study we present a mathematical model for QS in *S. meliloti*. It is based on molecular mechanisms that have been discovered by comparisons between wild-type and mutant strains and fits qualitatively with all features we measured using different genetic backgrounds. The most important foci of the model are a positive feedback which increases AHL production if the number of cells exceeds a certain threshold and a negative feedback for an even higher number of cells.

We stress that QS also poses a challenging evolutionary question: Since individual cells benefit from production of AIs by other cells, one might ask whether QS can be conserved during evolution. In other words, cheater cells which only use the AIs from other cells can be fitter because they do not invest in producing AIs. One way out is that only contributors to AI production can use the AI for downstream processes (Czaran and Hoekstra, 2007). Although interesting, such evolutionary questions are beyond the scope of the paper. However, a quantitative model for the molecular basis of QS will as well be helpful to decide the evolutionary stability of QS.

1. THE MODEL

Our goal is to provide a validated mathematical model for the Sin QS system in *S. meliloti*. After introducing the model, we give empirical evidence which shows how we came up with it. Afterwards we analyze the model and compare simulations with measurements.

1.1 The Full System

We introduce a minimal model for the molecular basis of quorum sensing, illustrated in Fig. 1. It consists of two ingredients: (i) a network of chemical reactions within each cell and (ii) the interaction between cells. The interaction between these two spatial scales is necessary for understanding the molecular mechanisms of quorum sensing. An essential ingredient is the signaling molecule which diffuses between cells. In our system the signal is an AHL. While in nature there is a huge variety of different chemical structures in AHL molecules, we model them as a single chemical species for simplicity. The AHL has two functions: firstly, it transports information between cells and secondly, it regulates the production of certain proteins within their host cells.

We describe the mechanisms of the two spatial scales in our system:

(i) The full network consists of the genes *sinR*, *sinI*, the corresponding proteins SinR and SinI, the protein ExpR, the signal AHL and the complex ExpR/AHL. Here, AHL is in either of two classes, within the cell (denoted AHL) and outside the cell (denoted AHL_o). The basic mechanisms of the full model are as follows (parameters α_j are rate for production, β_j are binding affinities to promoters and γ_j are degradation rates):

1. ExpR is a stable protein (no degradation is assumed in the model) and builds a complex with AHL at rate α_4.
2. The complex ExpR/AHL binds and unbinds to and from the binding site 3 upstream of *sinR* and 2 upstream of *sinI* at rates proportional to β_3 and 1, respectively, and can inhibit the expression of *sinR*. The complex is degraded at rate γ_4. The maximal production rate of SinR is α_1 and γ_1 is its degradation rate.
3. SinI is fully responsible for production of AHL at rate α_3. The promoter region of *sinI* has two binding sites, one for ExpR/AHL and one for SinR. SinR binds (to binding site 1 in Fig. 1) at a rate proportional to β_1 (relative to dissociation) and only when it is bound can production of SinI at rate α_2 begin. In addition, the complex ExpR/AHL can bind (to the binding site 2 in Fig. 1) at rate β_2 (relative to dissociation) and stimulate expression of *sinI*, leading to a maximal production rate $\alpha_2(1 + \alpha_5)$ for SinI. However, the latter is only possible if SinR occupies binding site 1.

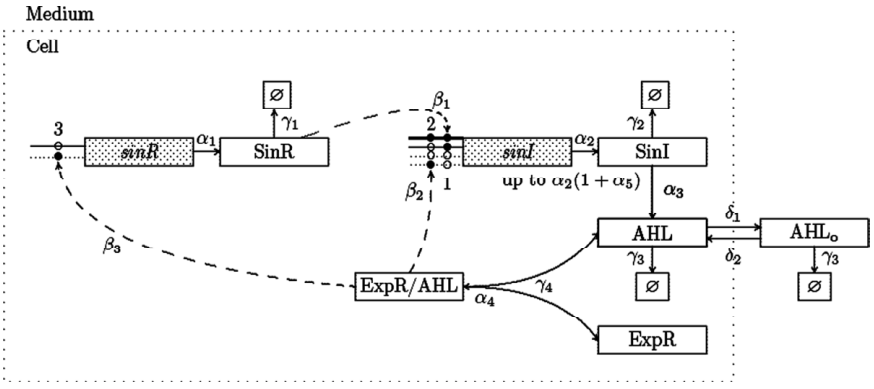


Figure 1: A summary of the molecular basis for the Sin quorum sensing system. Shaded boxes denote genes, other boxes denote proteins or AHLs, and Ø is a symbol for *nothing*. Each gene has its promoter and each promoter plus surrounding DNA sequence contains regulatory elements such as protein binding sites, which are denoted 1, 2, and 3. Unoccupied binding sites are denoted by ◦ and bound ones by •. Both promoters have different possibilities of being bound, indicated by the different horizontal lines. The thickness of the lines indicates promotor activity which is dependent upon the respective binding configuration. For example, the promoter of *sinI* is most active when both SinR and ExpR/AHL occupy their binding sites upstream of *sinI*. Solid arrows are reaction arrows, dashed arrows indicate binding to a promoter region, and Greek symbols denote reaction rates and binding affinities. For example, SinR is lost from the system (i.e., it degrades) at rate γ_1. The cell envelope is indicated by the rectangle with a dotted line, and AHLs are transported across the cell envelope.

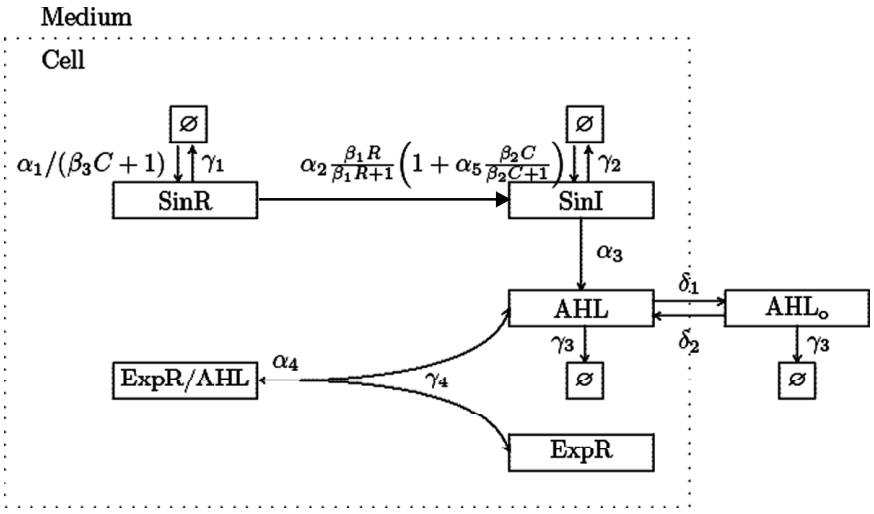
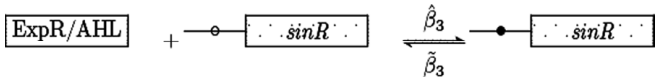


Figure 2: A simplification of the molecular system underlying quorum sensing. Arrows, boxes and reaction rates have the same meaning as in Fig. 1. Binding and unbinding to promoter regions is assumed to be in equilibrium at all times, leading to effective rates of production of proteins.

(ii) For the spatial component, AHLs are transported outside at rate δ_1 and to the inside of the cell at rate δ_2. We note that δ_2/δ_1 scales with the ratio of the volume of a single cell as compared to the volume of the medium. In addition, AHLs degrade at (small) rate γ_3.

1.2 Fast Binding and Unbinding to Promotor Regions

In order to make the system tractable, we assume that binding and unbinding rates of proteins to their DNA binding sites are rapid. In other words, (un)binding reactions are faster than all other reactions within the system. As an example, consider the chemical reaction, which is the binding of an ExpR/AHL-complex to the promotor region of sinR as part of the full system; see Fig. 1. Assuming that the number of ExpR/AHL molecules, C, is constant in the system (which is valid as long as binding and unbinding are fast reactions), we can model this reaction stochastically as follows: There are two Poisson-processes, $\hat{Y}$ with rate $\hat{\beta}_3 C$ and



$\tilde{Y}$ with rate $\tilde{\beta}_3$. If $\hat{Y}$ increases and if *sinR* is unbound, it is turned into the bound state. In addition, if $\tilde{Y}$ increases and if *sinR* is bound, it is turned into the unbound state. From this picture, it is easy to obtain the equilibrium probability for *sinR* being in the bound state. Assume that the system was started at time $-\infty$ and is observed at time 0. Then, *sinR* is bound at time 0 if and only if the last increase of the Poisson processes was made by $\tilde{Y}$. Since the rates $\hat{\beta}_3 C$ and $\tilde{\beta}_3$ were competing, it is a well-known property of exponential waiting times that

$$\mathbf{P}(\textit{sinR} \text{ unbound by time } 0) = \frac{\tilde{\beta}_3}{\hat{\beta}_3 C + \tilde{\beta}_3} = \frac{1}{\beta_3 C + 1},$$

with $\beta_3 = \hat{\beta}_3/\tilde{\beta}_3$. From the stochastic averaging theorem (Kurtz, 1992), and if $\hat{\beta}_3$, $\tilde{\beta}_3$ are very large, but their ratio converges to β_3, we conclude that to a good approximation, the full system behaves similar to a system where *sinR* is bound with probability $1/(\beta_3 C + 1)$ and unbound otherwise. As a consequence, let us look at the production of the protein SinR. In the full system, SinR is produced at rate α_1 if *sinR* is unbound and 0 otherwise. So, the effective rate of SinR production is

$$0 \cdot \frac{\beta_3 C}{\beta_3 C + 1} + \alpha_1 \cdot \frac{1}{\beta_3 C + 1} = \frac{\alpha_1}{\beta_3 C + 1}.$$

From this equation, we see that in a model with high binding and unbinding rates, it suffices to model the number of proteins SinR, SinI, ExpR as well as AHL and ExpR/AHL.

1.3 The Simplified System

The simplified model is obtained by using effective rates for production of proteins and the complex, if binding and unbinding to promotor regions is fast, as explained above. The resulting model (illustrated in Fig. 2) comes with the variables R_i (SinR in cell i), I_i (SinI in cell i), C_i (ExpR/AHL complex in cell i), A_{ii} (AHL in cell i) and A_o (AHL in the medium). Here, B is the total number of cells in the population. Since we assume that ExpR is not degraded, there is a constant $\bar{E}$ such that the number of molecules of ExpR is $\bar{E} - C_i$. The equations read

$$\begin{aligned}
\frac{dR_i}{dt} &= \alpha_1 \frac{1}{\beta_3 C_i + 1} - \gamma_1 R_i, \\
\frac{dI_i}{dt} &= \alpha_2 \frac{\beta_1 R_i}{\beta_1 R_i + 1}\left(1 + \alpha_5 \frac{\beta_2 C_i}{\beta_2 C_i + 1}\right) - \gamma_2 I_i, \\
\frac{dA_{ii}}{dt} &= \alpha_3 I_i - \delta_1 A_{ii} + \delta_2 A_o - \alpha_4(\bar{E} - C_i) - \gamma_3 A_{ii} + \gamma_4 C_i, \\
\frac{dA_o}{dt} &= B(\delta_1 A_{ii} - \delta_2 A_0) - \gamma_3 A_o, \\
\frac{dC_i}{dt} &= \alpha_4 A_{ii}(\bar{E} - C_i) - \gamma_4 C_i. \qquad (1)
\end{aligned}$$

This system of equations is analysed numerically for certain ranges of parameters below.

1.4 Empirical Justification of the Sin System Model

The advantages of modeling the Sin system include a relatively well understood molecular basis for transcriptional control and a relatively simple system coded by (only) three genes on one hand, and yet capable of responding appropriately to its environment and contributing significantly to the survival of the

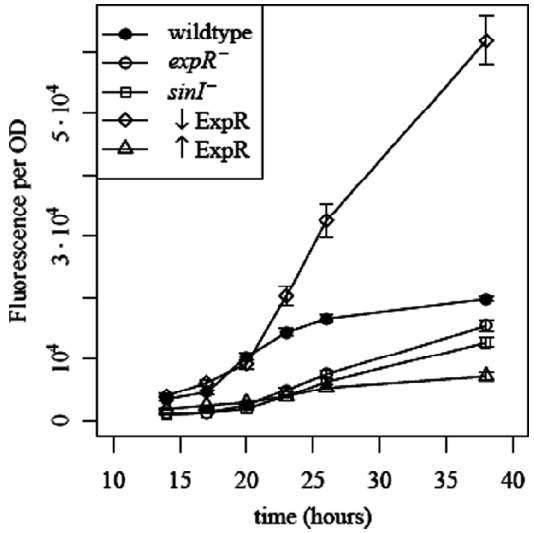


Figure 3: Fluorescence per OD over time as given by activity of the *sinI* promoter in various genetic backgrounds. The expR⁻ strain (Rm2011) carrying the vector construct pBSexpR is indicated by either ↑ExpR (induced expression of expR after addition of IPTG) or ↓ExpR (leaky expression of expR, no addition of IPTG).

bacterium on the other. The measurements we used to justify the equations (1) come from fluorescence data of the *sinI* promoter activity as given in Fig. 3.

QS requires a molecular basis which provides both a positive feedback loop and a negative feedback loop. The Sin system appears to be remarkably efficient at self-regulation by such feedbacks which are achieved when an AHL molecule forms a complex with ExpR. The positive feedback is switched on when a cell contains adequate amounts of the signaling molecule AHL. When the population reaches a high enough density, production of signaling molecules is no longer necessary and the system is switched off by a negative feedback. These two forms of regulation are both implemented in the system from Fig. 1. The positive feedback is activated when the ExpR/AHL binds to binding site 2 of the promoter region of *sinI* and increases expression of *sinI* which in turn increases production of AHLs. The negative feedback is seen when ExpR/AHL binds to the binding site 3 of the promoter region of *sinR* and inhibits expression of *sinR*, which leads to inhibition of *sinI* expression and thus a decrease of AHL production.

The Two Feedbacks

The ExpR/AHL complex is central for regulating the QS system. A short DNA region (≈40 nucleotides) within the 159 nucleotide *sinR-sinI* intergenic region was identified to be responsible for the regulation by ExpR (Bartels *et al*., 2007). Moreover, the specific DNA binding site sequence for ExpR/AHL was found by systematically exchanging each nucleotide within the 40 nucleotide region, narrowing the recognition sequence to 18 nucleotides (Fig. 5). The effect of ExpR/AHL binding to the *sinI* promoter region was a significant (3-fold) increase in *sinI* promoter activity, providing a positive feedback (McIntosh *et al*., 2008). However, the activity of the *sinI* promoter (and hence the positive feedback) is absolutely dependent on the presence of SinR, since disruption of *sinR* renders the *sinI* promoter inactive (McIntosh *et al*., 2008, 2009). SinR belongs to a class of regulators called the LuxR-type regulators, and a DNA sequence resembling a LuxR recognition site (tatgtacatgt) in the promoter region of *sinI* has been suggested (Bartels *et al*., 2007). In many AHL quorum sensing systems, the LuxR-type protein binds to and regulates the promoter of the gene coding for the AHL synthase. To test this, we fused various lengths of the 159 nucleotide *sinR-sinI* intergenic region (Fig. 5) to the promoterless reporter gene *egfp*. The variation in region length was due to a sequential removal of nucleotides from the promoter region, beginning with the nucleotides most distant from the *sinI* gene. Promoter activity was dependent upon the presence of various parts of the promoter region. For example, the loss of the ExpR binding site (94 nucleotides) corresponded with the loss of ≈ 70% activity, as was expected. Reducing the length of the region to 83 nucleotides did not further reduce activity, but the reduction to only 70 nucleotides resulted in the loss of > 95% activity. We note that the DNA sequence surrounding this site forms a perfect palindrome separated by 6 nucleotides, as indicated in Fig. 5. Also significant is that the second half of the palindrome matches the predicted LuxR recognition site. Furthermore, the transcription start of *sinI* has been identified at 27 nucleotides upstream of the start of *sinI* (McIntosh *et al*., 2008). This places the palindrome immediately upstream of a classical –35 promoter. Because this site is very close to the –35 region of a classical promoter, we cannot be sure that the removal of half the palindrome has not somehow destroyed the promoter. However, taken together, these observations fit well with the tentative conclusion that the palindrome is the SinR recognition site, and strengthens the conclusion that the presence of bound SinR is necessary for the activity of the *sinI* promoter.

The ExpR/AHL complex can also inhibit *sinR*, i.e., the complex negatively regulates *sinR* promoter activity through another specific DNA sequence in the DNA region upstream of *sinR*, providing a negative feedback loop (McIntosh *et al*., 2009). Both ExpR binding sites require AHLs for the full effect, and alterations of the DNA sequence within the binding sites resulted in corresponding loss of function. This finding was based on both a gel shift assay approach which measures the activity of purified components and by introducing the altered DNA into the cell and measuring its effect at a population level. The critical feature that determines which feedback loop dominates AHL production is the concentration of AHLs in

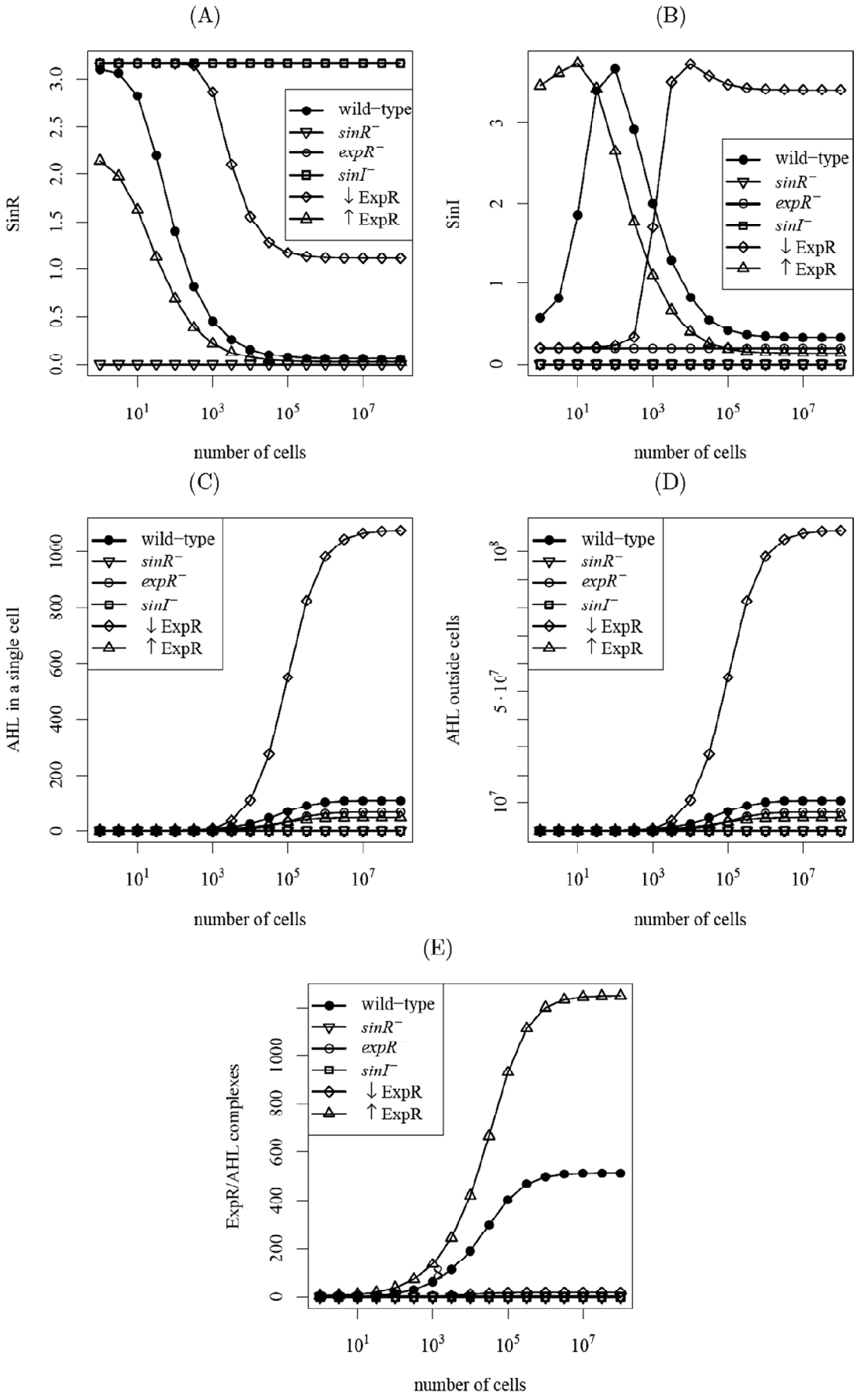


Figure 4: A simulation study of equation (1) with the parameters as given by (2). The *x*-axis is a proxy for the linear evolution of time, while abundances of molecules are given on the *y*-axis.

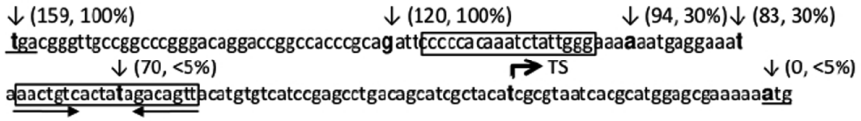


Figure 5: The 159 nucleotide region between *sinR* and *sinI*. This region as well as shorter fragments (indicated by the downward arrows) were fused to the reporter gene *egfp*. The numbers in brackets give the number of nucleotides in the fragment and the fragment activity relative to the full region, respectively. Underlined nucleotides represent the end of *sinR* (tga) and the beginning of *sinI* (atg). The bent arrow indicates the transcription start for *sinI*. The first (upper) box indicates the ExpR binding site (McIntosh *et al.*, 2008). The second (lower) box indicates the putative SinR binding site. This consists of a perfect palindromic repeat (aactgtc) separated by 6 nucleotides. Removal of the ExpR binding site results in a loss of 70% activity, while the removal of half the putative SinR binding site results in the loss of > 95% activity, as does the complete removal of the intergenic region.

the growth medium. At low AHL concentrations of 1-20 nM, the positive feedback loop dominates, resulting in the increased production of AHLs. As the AHLs accumulate to 50-100 nM, the negative feedback begins to decrease the rate of AHL production. Production continues, however, until the AHL concentration reaches ~1000 nM, at which point *sinI* promoter activity is completely inhibited (McIntosh *et al.*, 2009). Thus the positive and negative feedback mechanisms act in concert not only to quickly raise AHL levels, but also to prevent excess of AHL production. Extremely high AHL concentrations may drain energy sources or saturate the environment, rendering the quorum sensing signal obsolete.

Effects of Alteration of *expR* Expression on the *sinI* Promoter

The amount of the complex ExpR/AHL and thus the regulation of AHL concentration via SinI depends on concentrations of both ExpR and AHL. While AHL levels can be controlled externally, we also checked for the dependence of the activity of the *sinI* promoter and the production of AHLs on the level of ExpR; see also Figure 3. We used an inducible *E. coli lac* promoter in plasmid pBSexpR, which also expresses the LacIQ protein. Suppression of the *lac* promoter by LacIQ is not complete, which means that a small amount of ExpR protein is produced in the absence of the inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG). Addition of IPTG releases the *lac* promoter, resulting in the production of significantly higher levels of ExpR. When this plasmid was placed in a *S. meliloti* strain lacking a functional *expR*, leaky expression of *expR* (this mutant is denoted ↓ExpR) in the absence of IPTG resulted in a strong activation of *sinI* promoter activity. This activity was about 4-fold higher compared to the *sinI* promoter activity in the wild-type. However, when IPTG was added, releasing a strong expression of *expR* (↑ExpR), the *sinI* promoter activity responded by a 4-fold decrease compared to that in the wild-type. Strains lacking either *expR* or *sinI* exhibited a basal level of *sinI* promoter activity. This was about 3-fold lower than in the wild-type during earlier time points, but which eventually almost matched the wild-type activity at the last time point (30-40h). Taken together, these results indicate that lower levels of ExpR in the cell stimulate AHL production, while higher levels of ExpR repress AHL production. In particular, these experiments are in line with the presence of both a positive and a negative feedback in the wild-type strain. In the absence of either ExpR or AHLs, *sinI* expression is not regulated by quorum sensing. When *expR* expression is reduced, only few ExpR/AHL complexes are built. These are sufficient to bind to the *sinI* promoter region and enhance expression of *sinI*, but insufficient to inhibit *sinR* expression. When *expR* is over-expressed, many complexes are built. This results in the binding of the complex to the *sinR* promoter region and inhibition of *sinR* expression follows, resulting in less *SinI* and AHL production.

AHL Extraction and Detection

Fluorescence measured from the *sinI* promoter indicated that (i) a basal level of AHLs is produced if ExpR was missing, (ii) production of AHLs is up-regulated if the level of ExpR is low and (iii) production of AHLs is down-regulated if the level of ExpR is high. To confirm these predictions, AHLs were extracted from the supernatants of stationary phase cultures. Extracted AHLs from 0.5 mL of culture from the different

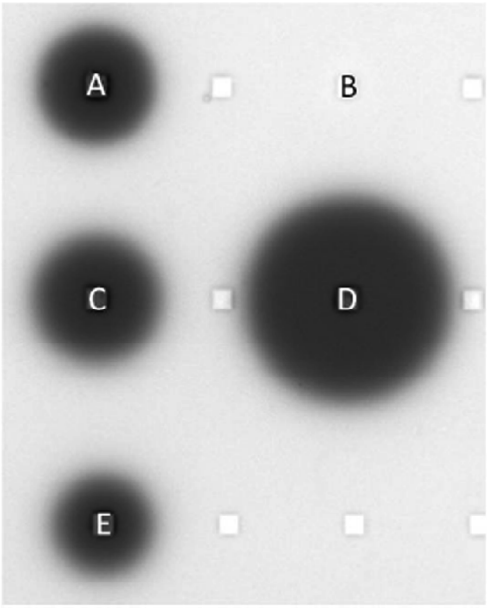


Figure 6: Spot test of AHLs extracted the supernatant of various *S. meliloti* strains using the *A. tumefaciens* indicator strain. Spots are as follows: A, 10 nmol AHL (C16: 1-HL); B, *sinI*⁻; C, wildtype; D, ↓ExpR; E, *expR*⁻. The development of color (blue) surrounding the spot is dependent upon the presence of AHLs. A stronger development indicates a higher amount of AHLs.

strains were spotted (5 μL) on an agar surface containing the indicator strain *Agrobacterium tumefaciens*; see Fig. 6. Color development by the indicator strain is an indication of the amount of AHLs in the extract. Extract from the *sinI*⁻ mutant contained no AHLs, while extract from the *expR*⁻ mutant contained levels slightly less than that of the wild-type. However, the ↓ExpR mutant with leaky expression from pBSexpR produced an obviously higher (> 4-fold) amount of AHLs than the wild-type.

Once again, the AHL extraction and spot test indicates that high levels of ExpR activate AHL production, while higher levels inhibit AHL production. This mirrors the effect of AHL levels previously observed with *sinI* promoter activity (McIntosh *et al*., 2009), where lower levels of supplemented AHLs to a *sinI*⁻ mutant strongly activated *sinI* promoter activity (by binding of ExpR/AHL to the promoter) and higher levels repressed *sinI* promoter activity (by inhibition of expression of *sinR* by binding to the promoter of *sinR*). Note that this explanation requires that the ExpR/AHL complex has a higher affinity for the Sin-system activating DNA binding site upstream of *sinI* than the Sin-system repressor site upstream of *sinR*. In particular, alterations of levels of ExpR provides another line of evidence that confirms the model which involves self-regulation based upon positive and negative feedback loops.

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Growth Conditions

S. meliloti strains were incubated at 30°C in a modified MOPS buffered minimal medium as described previously (McIntosh *et al*., 2009), which contained 48 mM MOPS (adjusted to pH 7.2 with KOH), 55 mM mannitol, 21 mM sodium glutamate, 1 mM $MgSO_4$, 250 μM $CaCl_2$ 37 μM $FeCl_3$, 48 μM H_3BO_3, 10 μM $MnSO_4$, 1.0 μM $ZnSO_4$, 0.6 μM $NaMoO_4$, 0.3 μM $CoCl_2$, 4.1 μM biotin and 0.15 μM K_2HPO_4. Cultures were grown in 100 μl volumes in 96 well plates, with at least 6 replicates, continuously shaken at about 1000 rpm. Where appropriate, antibiotics were added at the following concentrations: 120 $\mu g ml^{-1}$ neomycin, 10 $\mu g ml^{-1}$ nalidixic acid, and 10 $\mu g ml^{-1}$ tetracycline. For the monitoring of activity of the *sinI* promoter, we used a plasmid (pLK64) carrying the promoter region of *sinI* fused to the gene for green fluorescence protein (*egfp*) (McIntosh *et al*., 2008). Strains used in this study were also previously described, and include Rm2011 (*expR*⁻), Sm2B3001 (*expR*⁺, derived from Rm2011 as described in Bahlawane *et al*. (2008)), and

Sm2B4001 (*expR*$^+$, *sinI*$^-$, derived from Sm2B3001 as described in McIntosh *et al*. (2009)). For the controlled expression of *expR*, we used the vector pBSexpR (McIntosh *et al*., 2009). In this vector construct, *expR* was fused the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible *lac* promoter using the vector pSRK-Km (Khan *et al*., 2008). Induced expression of *expR* was via the inclusion of IPTG (0.5 mM) at inoculation of fresh medium, while leaky expression of *expR* was observed in the absence of IPTG.

2.2 EGFP Fluorescence Assay

Fresh minimal medium was inoculated from a preculture to an OD600 of about 0.004. Time is indicated in number of hours after inoculation. Background fluorescence (≈700-800 F/OD) was determined from strains carrying the pLK vector with a promoterless *egfp*, and has not been subtracted from values in Fig. 3, but was taken into account for the values presented in Fig. 5. The population density (OD_{600}) and EGFP fluorescence (excitation at 488 nm and emission at 522 nm) were measured using a Tecan Infinite M200 reader (Tecan Trading AG, Switzerland).

2.3 AHL extraction and spot test

For the extraction of AHLs, *S. meliloti* strains were grown until stationary phase in minimal medium. Supernatants were obtained after centrifugation and mixed 1:1 with ethyl acetate. The polar phase was removed and discarded, and the organic phase removed by rotary evaporation. The dried residue was dissolved in acetonitrile, at 1% of the primary volume.

The acetonitrile solutions of extracted AHLs were spotted (5 μl) on agar containing the indicator strain *A. tumefaciens* NTL4 (pZLR4) (Zhu *et al*., 2003) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the bioassay was incubated at 30°C for 12 hours.

2.4 Simulations

In order to obtain a quantitative picture of the model from equation (1), we relied on simulations using the software **R**. For a series of different cell numbers *B*, we used the package **rootSolve** to find the steady state of the system. We checked numerically that low and high AHL initial concentration led to the same stationary point.

3. ANALYSIS OF THE MATHEMATICAL MODEL

3.1 Qualitative Fit to Data

The model as given by the equations (1) is only qualitative, since we have not yet specified any parameters. The reason is that we cannot directly compare outcomes from the model with empirical measurements. However, we can in fact estimate orders of reaction rates such that the system shows all features of a functioning QS system.

We assume that the number of cells *B* is given as an external parameter. Equivalently, reactions within each cell are in equilibrium at the time the cell divides. When the cell replication rate is approximately constant – which is a reasonable assumption in the early phase of QS – the *x*-axes of Fig. 4 are a proxy for the linear evolution of time.

In our simulation results in Fig. 4, we used the following parameters for the wild-type and $N = 10$:

$$\begin{aligned}
&\alpha_1 = N^{3/2}, && \alpha_2 = N^{1/2}, && \alpha_3 = N^{3/2}, && \alpha_4 = N^{-3}, && \alpha_5 = N^{3/2}\\
&\beta_1 = N^{-1/4}, && \beta_2 = N^{-1/2}, && \beta_3 = N^{-1},\\
&\gamma_1 = N, && \gamma_2 = N, && \gamma_3 = N^{-1}, && \gamma_4 = N^{-1},\\
&\delta_1 = N^3, && \delta_2 = N^{-2}, && \overline{E} = N^3.
\end{aligned} \tag{2}$$

The absolute numbers are less important for our simulations than their orders of magnitude. We explain the qualitative choice of the parameters as follows:

- *The QS system must be able to quickly react to an external signal:* If QS helps the population to sense the environment and therefore react to limited nutrition or other signals, the QS system must be able to react quickly to an external stimulus. For this reason, the degradation rate γ_2 of SinI must be high. Otherwise, SinI would continue producing AHLs although the environment of the cell changed. The same argument applies to the degradation rate γ_1 of SinR, because SinR regulates the transcription of *sinI*. (Interestingly, in our group, extremely high degradation rates of SinR and SinI have recently been observed, Stefan Meyer, personal communication.) The production and degradation rates of SinR and SinI must be appropriate to allow for both positive and negative feedback mechanisms.
- *The positive feedback must be a reaction to the presence of more cells:* In other words, a single cell in a large volume is not able to enter a positive feedback loop. To see how this works in our system, look at a single AHL molecule which is just created within cell *i*. Either it builds a complex (at maximal rate $\alpha_4 \bar{E}$, if no complex has been built yet) or it is transported out of the cell (at rate δ_1). We assume that $\delta_1 \gg \alpha_4 \bar{E}$. In that case, the AHLs produced by a single cell in a large volume exit the cell and are lost from the system without forming any ExpR/AHLs complexes which would lead to the positive feedback. When many cells are present and AHLs also enter the cell by the rate δ_2 (which is much lower than δ_1 since the volume inside a cell is relatively small), the positive feedback can start.
- *Both, the positive and the negative feedback loop must be reached:* When an ExpR/AHL complex is built, it can either bind to the promoter region of *sinR* or to the promoter region of *sinI*. We assume that it is more likely to bind to the promoter region of *sinI*. Then, if the promoter region of *sinI* is bound, but the promoter region of *sinR* is still unbound, expression of *sinI* is increased and a positive feedback is activated. This means we assume that $\beta_2 \gg \beta_3$. See Fig. 7 to see the expression of *sinI*, which is proportional to AHL production for a cell in all three states, i.e., the basic, positive and negative feedback states.
- *Both AHL and the complex ExpR/AHL are stable, but still degrade at a low rate:* In order to reach a proper equilibrium, we assume a small degradation rate of AHLs and a small disassembly rate of the complex ExpR/AHL into ExpR and AHL.

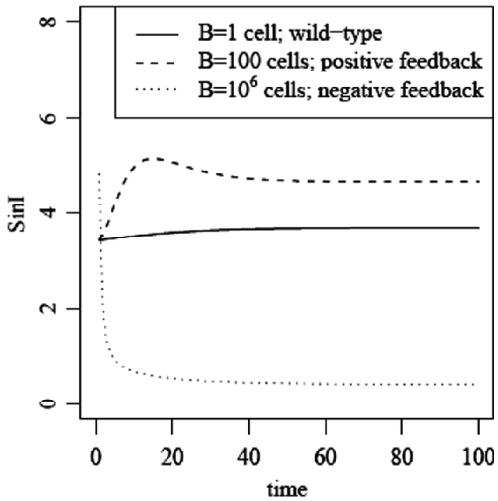


Figure 7: The abundance of SinI depending on the number of cells. For $B = 10^2$, a positive feedback is seen, and $B = 10^6$ leads to an inhibition of *sinI* as compared to the wild-type.

3.2 Behavior of Mutants

Compare Fig. 3 and Fig. 4(D). Since Fig. 3 gives observations for the activity of the promoter of *sinI*, and SinI is fully responsible for AHL production, these two figures should show the same behavior. As seen in both figures, the ↓ExpR mutant produces most AHLs, and the wild-type amounts to a higher AHL concentration than the ↑ExpR mutant. Only the *sinI*⁻ mutant shows a different behavior; the reason is that the measurements indicate activity of the promoter rather than the AHL production which is null if SinI is not present. We give a more detailed view of the mutants:

- *Behavior of the sinR⁻ mutant* (In the simulations we used $\alpha_1 = 0$): In the absence of SinR, binding site 1 of *sinI* cannot be occupied and so, *sinI* cannot be expressed, leading to no AHL production. This behavior was experimentally seen previously in a *sinR*⁻ strain (McIntosh *et al*., 2008, 2009).
- *Behavior of the expR⁻ – mutant* (In the simulations we used $\bar{E} = 0$): In the absence of ExpR, the complex ExpR/AHL cannot be built. Therefore, SinR and SinI production only occur at a basal rate, and neither positive feedback (the amount of SinI does not change with a growing number of cells) nor negative feedback (SinR does not change with a growing number of cells) can lead to a change in the production of AHL.
- *Behavior of the sinI⁻ mutant* (In the simulations we used $\alpha_2 = 0$): In the absence of SinI, no AHLs can be produced, and thus no ExpR/AHL complex can be built. Only SinR molecules are generated.
- *Behavior of the ↓ExpR mutant* (We lowered $\bar{E}$ by a factor of 50): While the *expR*⁻ mutant shows only a basal promoter activity of *sinI* (or production of AHLs), *sinI* promoter activity of the ↓ExpR mutant is increased relative to the wild-type. The reason in our mathematical model is that the amount of ExpR protein is large enough to produce ExpR/AHL complexes such that the positive feedback can operate, but is too small for a production of ExpR/AHL molecules which bind to the *sinR* promoter and lead to the negative feedback.
- *Behavior of the ↑ExpR mutant* (We increased $\bar{E}$ by a factor of 4): If ExpR levels are increased, the rate of production of the ExpR/AHL complex is increased. For this reason, the negative feedback is entered quicker, i.e., at a lower cell density.

3.3 Steady States

Since the system (1) is non-linear, there is a chance of bi-stability. Of particular importance for each cell is the number of AHL molecules, since they trigger both the positive and the negative feedback loop. Moreover, it has been shown in various systems of QS that bi-stable chemical reaction networks within cells can lead to different behavior of different cells. Note that (by our assumption of a well-stirred system) all cells share the same number of AHL molecules in the medium. So, we want to analyze the dependence of A_{ii} and A_o for the system (1) in equilibrium.

Now assume that R_i, I_i and C_i are in steady states. This means we set the left-hand sides in (1) to zero and calculate the steady state depending only on A_{ii}. This leads to

$$C_i = \frac{\bar{E}}{\frac{\gamma_4}{\alpha_4} + A_{ii}} A_{ii}$$

$$R_i = h_1 \frac{\frac{\gamma_4}{\alpha_4} + A_{ii}}{h_2 + A_{ii}} A_{ii}$$

$$I_i = h_3 \frac{\frac{\gamma_4}{\alpha_4} + A_{ii}}{(h_4 + A_{ii})} \left(1 + h_5 \frac{A_{ii}}{(h_6 + A_{ii})}\right) \tag{3}$$

with

$$h_1 = \frac{\alpha_1}{\gamma_1(\beta_3 \bar{E} + 1)}, \qquad h_2 = \frac{\gamma_4}{\alpha_4(\beta_3 \bar{E} + 1)}, \qquad h_3 = \frac{\alpha_2 \beta_1 h_1}{\gamma_2(h_1 \beta_1 + 1)},$$

$$h_4 = \frac{h_2 + h_1 \beta_1 \frac{\gamma_4}{\alpha_4}}{h_1 \beta_1 + 1}, \qquad h_5 = \frac{\beta_2 \alpha_5 \bar{E}}{\beta_2 \bar{E} + 1}, \qquad h_6 = \frac{\gamma_4}{\alpha_4(\beta_2 \bar{E} + 1)}.$$

This means that in equilibrium we have the relationship

$$0 = \frac{dA_{ii}}{dt} = \frac{\alpha_3 h_3 \left(\frac{\gamma_4}{\alpha_4} + A_{ii}\right)}{(h_4 + A_{ii})} \left(1 + h_5 \frac{A_{ii}}{(h_6 + A_{ii})}\right) - \delta_1 A_{ii} + \delta_2 A_o - \gamma_3 A_{ii}. \tag{4}$$

It can easily be seen that there is a one-to-one relationship between A_{ii} and A_o and no bi-stability is possible; see Fig. 8 for an illustration. Moreover, by our choice of parameters, this equilibrium is dominated by transport into and out of the cell, i.e., approximately

$$\delta_2 A_o = \delta_1 A_{ii}, \tag{5}$$

holds. For this reason, the linear relationship in Fig. 8 is explained.

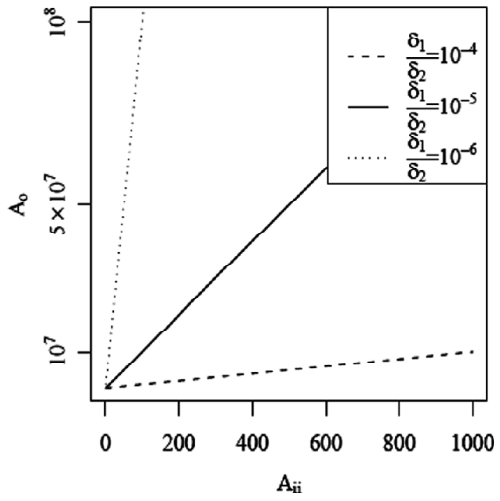


Figure 8: In equilibrium, the number of AHLs within a single cell, A_{ii}, and out of cells, A_o, from (4), is displayed. The parameters from (2) were used, and δ_2 was changed for the three choices of δ_1/δ_2.

3.4 Stochastic Simulations and Sensitivity for $\bar{E}$

In addition to the deterministic modeling approach using the system (1), we performed stochastic simulations using the Gillespie algorithm (Gillespie, 1976). It has been argued that stochastic simulations should be used when the copy number of a chemical species is low, which is in our case true for SinR and SinI.

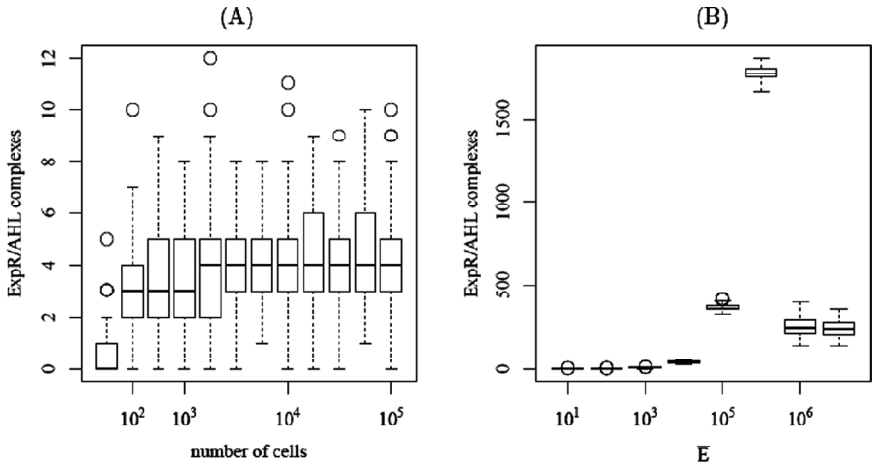


Figure 9: In stochastic simulations, the number of ExpR/AHL complexes is different from the deterministic system (compare with Fig. 4E) for many parameter combinations. The reason is the high sensitivity of both systems to the total number of ExpR molecules, $\overline{E}$.

However, we see the greatest difference in the deterministic versus stochastic simulations in the number of ExpR/AHL complexes; see Figure 9(A), which shows abundances of the complex in the wild-type for various cell numbers. While the deterministic system (see Fig. 2(E)) shows up to 500 complexes for the same parameters, the stochastic system only comes with around 5 such molecules. Investigating this difference, it turns out that the system is highly sensitive to the total number of ExpR molecules (i.e. to the parameter $\overline{E}$ in (1)). This behavior was already apparent in Figure 2(E). The three curves representing the wild-type, ↓ExpR and ↑ExpR show a strong diversity in their development. ↓ExpR means that less ExpR molecules are present in the cells as in the wild-type cell and ↑ExpR denotes the case where the number $\overline{E}$ is raised compared to the wild-type. Hence, due to fluctuations in the number of complexes, which might be triggered by bursts of production of AHL molecules, the chance that a cell in the stochastic system comes with many complexes is low, unless the abundance of cells is high; see Fig. 9(B).

4. DISCUSSION

Based on empirical evidence, we present a mathematical model for quorum sensing in the nitrogen fixing bacterium *S. meliloti*. The system comes with the players, SinR, SinI and ExpR, as well as the signaling molecule AHL. Our mathematical model from equation (1) differs from other approaches for a quantitative understanding of QS in several respects. In the models from Dockery and Keener (2001), Müller *et al.*, (2006), and Williams *et al.*, (2008), cells host bi-stable chemical systems: if initial production of AHL is low, the complex is built not often enough for entering the positive feedback. However, if many AHLs are within the cell initially, the AHLs are more probable to build the complex rather than to exit the cell. Consequently, the positive feedback can be entered. For a bi-stable system, Williams *et al.*, (2008) report that the population consists of a bi-modal distribution, centered around cells which are in the basal state and those in the positive feedback state. In contrast to the above-mentioned approaches, our model for QS in *S. meliloti* does not require a bi-stable molecular basis within each cell. Rather, the total number of cells controls the biochemical system within single cells. If the density of cells is low, most signaling molecules are lost to the environment. At higher cell density, the signaling molecules may re-enter the cell, build a complex with ExpR, and bind to the *sinI* promoter region which results in a positive feedback. At even higher cell density, even more AHLs are available in the medium, so that AHL levels within cells also increase. A negative feedback is obtained when sufficient levels of the ExpR/AHL complex are reached, and this complex binds to the *sinR* promoter and inhibits transcription of *sinR*.

The presence or absence of a bi-stable chemical mechanism within cells is not the only difference to existing models of QS. In *V. fischeri*, the QS system consists of two (called LuxI and LuxR) rather than

three proteins in *S. meliloti* (SinR, SinI and ExpR). Most models for QS in *V. fischeri* concentrate on the positive feedback loop, and do not model a negative feedback loop, e.g. Müller *et al.*, (2006). However, our results indicate that three players are essential in the QS system of *S. meliloti*, and therefore we require a more complex model. A model of similar complexity was found in *P. aeruginosa* (Kuttler and Hense, 2010), which also comes with both, a positive and a negative feedback. However, the molecular explanation for both types of feedback is quite different: Our system enters the positive feedback when the ExpR/AHL complexes binds to the *SinI*-promotor, and a negative feedback when the same complex binds to the SinR promotor. In the RsaL-system of *P. aeruginosa*, the positive feedback is entered, when the PpuR/AHL complex binds to ppu, a promotor triggering AHL production, and a negative feedback if RsaL – a protein which negatively controls its own expression – binds to the same ppu and therefore suppresses AHL production. So, in *P. aeruginosa*, the two feedback loops only require a single promoter (ppu), while the system of *S. meliloti* appears to be more complex in this respect.

A critical assumption in our system is that transport of AI molecules across the cell wall is not limiting. (In order to see this from (1) and the parameters used in (2), note that the reaction rate δ_1 of the transport, is higher than any other reaction rate for AHL.) Such a diffusive behavior was already shown in Kaplan and Greenberg (1985) for the AIs used by *V. fischeri*. However, the AIs in *S. meliloti* are long-chain AHLs, while *V. fischeri* uses smaller and more soluble analogues. Recent evidence suggests that while short-chain AHLs appear to diffuse through the double membrane barrier of Gram-negative bacteria, the long-chain AHLs are suspected to be assisted by active transport (Pearson and Iglewski, 1999; Chan *et al.*, 2004; Mima and Schweizer, 2010). However, the players or process involved remains almost completely unknown.

Although we could not estimate model parameters within the QS system of *S. meliloti*, the mathematical model correctly predicts the behavior for mutants lacking ExpR or SinI, and the response to a down- or upregulation of *expR* expression. When enough quantitative data is available, both on the wild-type and mutants, we will provide a quantitative fit to the system (1).

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