Quorum sensing in *Sinorhizobium meliloti*: 
AHL-induced expression of *sinI*

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Abstract

*Sinorhizobium meliloti* is a species of quorum-sensing bacteria that has a symbiotic relationship with its plant host, *Medicago sativa*. Quorum sensing allows bacteria to communicate by excreting and uptaking chemical signals, called autoinducers, and change the behavior of the entire population once a critical concentration of autoinducer is reached. *S. meliloti* is predicted to have at least 2 quorum sensing networks (QSNs): the hypothesized Mel system and the known Sin system. The Sin QSN has a synthase (SinI) which produces several long-chain AHLs with a wide range of carbon-chain length (C$_{12}$ to C$_{18}$). Though it has been studied for a while, not much is known about the specifics of the Sin QSN. In this paper, we test the effects of several AHLs (C$_4$-C$_{18}$) on various strains of *S. meliloti* to see if more can be understood about the complex behavioral network in these small bacteria.

1 Introduction

Quorum sensing (QS) is a process through which bacteria communicate with each other [1]. It is facilitated by three main parts: (1) autoinducers, which are the chemical signals used by the system for effective communication; (2) synthases (which produce the chemical signals); and (3) receptors (which bind to the autoinducers). In ram negative bacteria, like *Sinorhizobium meliloti*, N-acyl homoserine lactons (or AHLs) are the preferred autoinducer. The bacteria "learn" about and respond to environmental changes by producing, emitting, binding to, and responding to these autoinducers. This system allows for population-wide behavioral changes, which are essential for survival. Uncovering the mechanisms used by different quorum sensing networks (QSNs) will allow us to better manipulate the behavior of harmful or benefi-
Figure 1: The well-known QSN of *V. fischeri*. LuxI is a synthase that produces the autoinducer, 3-oxo-C$_6$. It is released from the cell, where more AHLs have been excreted. It is then taken in by a cell and attaches to LuxR, the receptor protein. These two together bind to the "lux-box" (an operon with several genes including luxI and the gene for luciferase) and promote the production of luciferase, the bioluminescent protein. In addition, luxI is also promoted, and thus creates a positive feedback loop that accelerates the quorum sensing process.

at the center of QS research on *S. meliloti*. The Sin system is essential for symbiosis with the host plant; strains without a functional synthase cannot successfully invade the host plant’s roots [4]. In addition, *Medicago sativa* produces AHL-like signals that can interfere with the activities of the Sin QSN [5].

Three proteins work within the framework of the Sin system: SinI, SinR, and ExpR [6, 3]. Sin I is a LuxI homolog; i.e., SinI is the synthase which produces the autoinducers used by the system. The position of sinR with respect to sinI on the genome (directly upstream; see Figure 2) would imply that SinR is a LuxR homolog [7]; however, it has been shown that ExpR is the protein that actually binds to the AHLs produced by SinI [4]. Though ExpR is an LuxR solo (expR is not near sinI on the bacterial genome) [8], it does have a binding site is located in the intergenic space between sinI and sinR [7].

Several AHLs have been shown to be used by the rhizobia. Of these, those with carbon chains of lengths between 12 and 18 have been shown to be synthesized by the Sin system; though there is evidence of other AHLs being produced by the bacteria [6]. This wide range of carbon-chain length is rather peculiar; these lengths correspond directly to the ability of the AHL to diffuse. Though much is known about *S. meliloti*, a lot is still left to discover, such as what the purpose of sinR is, whether SinR interacts with Sin AHLs, and whether all the AHLs predicted to be produced by SinI are actually used by the Sin QSN. Once this is known, the bigger purpose of this experiment may be carried out: to discover the diffusion patterns of AHLs in the bacteria.

In this paper, we try to seek the answers to these questions. Our experiment testing the purpose of SinR is running and will provide answers within the next week, but we have shown that of the many AHLs produced by SinI, only 3-oxo-C$_{14}$, C$_{16:1}$, and 3-oxo-C$_{16:1}$
Figure 2: The arrangement of the quorum-sensing-related genes in the *S. meliloti* chromosome. It has been predicted that the ExpR binding site is in the intergenic region between *sinR* and *sinI*. The *lux-box*-like gene is predicted to be the binding site for SinR.

change the response behavior of the sinI mutant strain when grown in Ty medium.

2 Methods and Materials

The fluorescence and optical density curves were produced by using a BioTek machine. Gen5 software was used to program the machine to take measurements of the optical density and fluorescence every 10 minutes for 48 hours. Polystyrene 48-well plates and 96-well plates were primarily used in these experiments.

Five strains of bacteria were studied in these series of experiments: 8530 (wild type), 1021 (*expR* mutant), MG32 (*sinI* mutant), MG75 (*sinI, expR* double mutant), and MG170 (*sinR* mutant). Each of these strains contained a *psinI-gfp* plasmid—i.e., when the *sinI* promoter was activated (*psinI*), GFP would fluoresce green when blue light was shone on it. Glycerol stock of these strains were kept in a -80 °C freezer.

Ice from the glycerol stock cultures was scraped onto a pipette tip and dropped into 5 mL of Ty medium and grown overnight (around 14 hours) in a 30 °C incubator. These new cultures were used to streak plates of 1.5% agar (made with antibiotics streptomycin at 400 µg/mL and spectinomycin at 50 µg/mL) and then incubated at 30 °C until enough colonies were formed, but before to many colonies started to merge. These agar plates were used for several experiments, and then retired when colonies from a single clone were no longer able to be picked. Clonal colonies were picked with a pipette tip and dropped into 5 mL of Ty and grown overnight (around 9 hours) until the optical density was between 0.2 and 0.4. These colonies were diluted 100-fold, returning them to an OD between 0.002 and 0.004, so the fluorescence due to expression of the *sinI* promoter could be seen at all stages of growth.

The autoinducers were treated in four ways:

1. Originally, 400 µL of diluted bacteria were added to each well. The AHLs (stored in ethyl acetate) were added directly to the bacteria in the well plates after the bacteria were placed in the wells; however, as ethyl acetate kills bacteria and the effects from this were noticed, it was decided that a new method needed to be used.

2. Phosphate buffer dilutions were used next: The AHLs in ethyl acetate were evaporated in polycarbonate tubes, leaving just the AHLs. Phosphate buffer was then added to the appropriate concentration and mixed thoroughly. Since AHLs do not mix well with phosphate buffer, the concentration of the AHLs was doubted.

3. Further experiments were conducted by placing the AHLs directly into the well plate, and then evaporating. Since the ethyl acetate ate up the polystyrene, 150-200 µL of deionized water were added to the bottom of each well, followed by pipetting 4 µL of the autoinducer-ethyl acetate mixture on top of the water. The wells were placed in a 60 °C oven and left to evaporate until all the liquid was gone from the wells. As the ethyl acetate is lighter and evaporates more quickly, the water was a pro-
Table 1: The various names and characteristics of the strains studied in these experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristics</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Rm8530</td>
<td>1021 expR+ SmR</td>
<td>Pellock et al., 2002</td>
</tr>
<tr>
<td>Rm1021</td>
<td>expR102::ISRm2011-1 expR, SmR</td>
<td>Galibert et al., 2001</td>
</tr>
<tr>
<td>MG32</td>
<td>8530 δsinI, SmR</td>
<td>Gao et al., 2005</td>
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<tr>
<td>MG75</td>
<td>1021 δsinI, SmR</td>
<td>Gao et al., 2005</td>
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<tr>
<td>MG170</td>
<td>8530 δsinR, SmR</td>
<td>Gao, et. al, 2012</td>
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4. Final experiments were conducted by diluting the AHLs with ethanol. Ethanol does not eat away polystyrene, and so did not have a negative affect on the well plates. The AHLs were serially diluted in ethanol, and 4 µL of the diluted solution was added to the appropriate wells. The well plate was left under the hood until all the ethanol was evaporated. Ty and the bacteria were then added to the evaporated wells, and mixed with a pipette.

After adding both autoinducers and bacteria to each of the wells, the well plates were placed in the BioTek machine and data was collected for 48 hours at 30 °C.

Two experiments were conducted testing the effects of phosphate on psinI activity. The first experiment used a 100 mM phosphate buffer and Ty medium mixture. The second experiment used a 2 mM phosphate buffer and Ty medium mixture. To add phosphate to the Ty medium, a 1 M solution of phosphate buffer at a pH of 7 was diluted using Ty medium to the appropriate concentrations. The various strains were then grown in this mixture.

Furthermore, to test the purpose of SinR, the psinI-gfp plasmid and a plasmid containing sinR were transformed into E. coli cells via electroporation. The results of these experiments have not yet been delivered, so further detail will be provided when the results come in.

3 Results

In our experiments, we see that only MG32 (sinI mutant) has any response to a variation in the concentration of AHL (Figure 3); it is the only one of the strains not producing AHL (it lacks sinI) but also has expR (the receptor). The psinI activity in MG32 is significantly lower than in MG75, as shown in Figure 7. However, with the right AHLs, psinI activity increases in MG32, with 3-oxo-C16 raising psinI activity to a level greater than the MG75. The C16 and 3-oxo-C14 raise the psinI activity, but not enough to overcome the psinI activity of MG75. As Figure 3 and Figure 5 show, of all the AHLs believed to be produced by the Sin system (C12-C18), only 3-oxo-C16, C16, and 3-oxo-C14 showed any increase in psinI activity in the MG32 strain.

Looking at Figure 3 and Figure 4, we see that none of the AHLs have any effect on psinI expression in MG75, the only other strain that does not produce its own AHLs. Furthermore, MG75 (sinI, expR double mutant) and 1021 (expR mutant) have fluorescence curves that increase continuously despite the leveling-off of the optical density.

The most significant impact on psinI expression aside from removing sinI itself was
Figure 3: sinI promoter activity of various strains of *S. meliloti* with all AHLs. 8530 is the wild-type strain with *psinI-gfp*; 1021 is the expR mutant with *psinI-gfp*; MG32 is the *sinI* mutant with *psinI-gfp*; MG75 is the *sinI, expR* double mutant with *psinI-gfp*; MG170 is the *sinR* mutant with *psinI-gfp*. It is important to note that data from the 8530 is not reliable; as it forms EPS, a colony forms at the bottom of the wells, making the fluorescence hard to read. Fluorescence curves of the 8530 (not shown) show the eccentric, and hard to map behavior of the 8530.
Figure 6: sinI promoter activity of 1021 (expR mutant) and MG75 (sinI, expR double mutant) with various AHLs (C_{12}, 3-oxo-C_{14}, 3-oxo-C_{16}, C_{16:1}, and C_{18}). The two have identical fluorescence curves, in spite of the fact that MG75 does not have a functional sinI.

Figure 7: sinI promoter activity of MG32 (sinI mutant) and MG75 (sinI, expR double mutant) with various AHLs (C_{12}, 3-oxo-C_{14}, 3-oxo-C_{16}, C_{16:1}, and C_{18}). The psinI activity of MG75 is significantly greater than that of MG32, unless an autoinducer binds to ExpR.
Figure 4: sinI promoter activity of MG75 (sinI, expR double mutant) with several long-chain AHLs, with AHLs prepared by way of method 4. MG75 does not respond to any of the AHLs.

Figure 5: sinI promoter activity of MG32 (sinI mutant) with several long-chain AHLs, with AHLs prepared by way of method 3. The concentration of AHL needed for a response is extremely high (100 µM).

Figure 9: sinI promoter activity of MG32 (sinI mutant) with long-chain AHLs, with AHLs prepared by way of method 4. The concentration of AHL needed for a response is lower (1.5 µM).

removing sinR, with around a 3-fold decrease in fluorescence. Disrupting expR reduced the fluorescence by a factor of two. Once expR was removed, disrupting sinI from the expR mutant had little to no effect; the expR mutant and expR, sinI double mutant had the same fluorescence curves (Figure 6). As this result was surprising, PCR was done to confirm that these two strains were in fact different; the results showed that they were.

As the experimental technique improved, the concentration of AHL required to induce a response decreased significantly (compare Figure 5 and Figure 9). The final experiments show a response to concentrations around 150 nM.

When the phosphate concentration was increased to 2 mM, no effect was detected; however, when the phosphate concentration was increased to 100 mM, a significant change in the behavior of the bacteria was detected. The fluorescence curves had an interesting pattern (Figure 8).
4 Discussion

The main interest of our physics group in *S. meliloti* has to do with the wide range of AHLs it produces. As this group previously did an experiment looking at the QS diffusion patterns of *Vibrio fischeri*, we thought it would be interesting to see how an organism interacted with a wide range of autoinducers. Our hypothesis was that the short-chain AHLs would diffuse more quickly and over a larger area than the long-chain AHLs, and thereby control bacterial population behavior on a grand scale, while the long-chain AHLs would control behavior at a more local range. However, in the process of learning more about this species, we have discovered that there is much left to understand about the interesting quorum sensing network of these bacteria before diffusion experiments can be conducted.

Marketon *et al.* (2002) identified six AHLs being produced by the Sin QSN: C₈-HL, C₁₂-HL, 3-oxo-C₁₄, 3-oxoC₁₆ : 1, C₁₆:₁, and C₁₈ [6]. They also discovered AHLs with shorter chains: 3-oxo-C₆ and C₆; however, these AHLs were unaffected by a disruption in the *sinI* gene, implying that the Sin QSN does not produce these AHLs. Teplitski, *et al.* (2003) found a response from C₁₄, 3-oxo-C₁₄, C₁₆, 3-oxo-C₁₆ and C₁₆:₁, but did not find a response from C₈, C₁₂, or C₁₈ [9]. We only found a response in 3-oxo-C₁₄, 3-oxo-C₁₆ and C₁₆:₁. It should be noted that the environment of the rhizobia has a profound effect on the way that these bacteria interact [10]; the Teplitski group found a response from C₁₄-HL and C₁₆-HL in different growth media than the one used in this experiment (Ty).

We are unsure of why *S. meliloti* would produce so many AHLs without using all of them. What is clear, however, is that the primary AHLs seem to be 3-oxo-C₁₄, 3-oxo-C₁₆:₁, and C₁₆:₁ in that order. This is further supported by an experiment conducted by Gao *et al.* (2007), in which a gene encoding for AiiA (which inactivates AHLs) was inserted into Rm1021 [11]. The exper-
Figure 10: SinI produces several AHLs; of these, only 3-oxo-C\textsubscript{14}, C\textsubscript{16:1}, and 3-oxo-C\textsubscript{16:1} bind to ExpR, which represses sinR activity, but promotes sinI activity when bound to AHL. SinR constantly upregulates sinI, but is repressed by ExpR and promoted by PhoB. A positive feedback loop with expR allows for the autoregulation of expR.
iment showed that without AiiA, 3-oxo-C\textsubscript{16:1} was shown to be the most abundant QS signal, followed by C\textsubscript{16:1} and C\textsubscript{16} (in minimal medium), and finally 3-oxo-C\textsubscript{14}. With the AiiA insertion, however, C\textsubscript{16:1} was the only AHL detected, with its signal only reduced by 90%. At these levels, this paper also showed that even at these low concentrations of AHL, the bacteria still swarmed, indicating that this AHL allows for quorum sensing in even the direst circumstances.

Not much is known about SinR; it is not very soluble. McIntosh et al. (2009) showed that eliminating sinR reduces sinI expression to background levels, while the overexpression of sinR increases sinI expression 8-fold, implying that SinR is the primary promoter of sinI [10]. Looking at the fluorescence of the sinR mutant in our experiments, we see the same thing; the fluorescence drops down to the same levels as the sinI mutant. However, looking at Figure 3, and considering the fact that sinI and sinR transcribe in the same direction, there is the possibility that disrupting sinR also disrupts sinI. As Figure 3 and Figure 5 show, eliminating expR increases psinI activity two-fold (compare the sinI mutant with the expR mutant and sinI/expR double mutant). Since SinR seems to be the biggest promoter of sinI [10], this implies that ExpR represses sinR expression somehow, an idea suggested by McIntosh et al. (2009).

Comparing MG32 with the other strains in Figure 5, we see that while disrupting sinI reduces psinI activity to background levels, activity is regained with the addition of AHL. This implies that ExpR regulates itself. Our experiments show that without expR, we see no response in psinI activity due to increased AHL concentrations. Furthermore, looking at Figure 6, we can see that once expR has been disrupted, removing sinI has no effect on the activity of psinI; i.e., ExpR is necessary for AHL-dependent quorum sensing.

We see that phosphate has some clear effect on the system. McIntosh et al. (2009) showed that in phosphate-limiting conditions, sinR is significantly induced [10]. This dependence of sinR on phosphate levels is mediated through the Pho regulon. One study found that there are around 96 Pho boxes in the \textit{S. meliloti} genome [12]. Another study located one Pho box upstream of sinR [13].

Putting all of this together, we can organize a new circuit diagram for \textit{S. meliloti} (Figure 4). The next step for this experiment (once we know which autoinducers create a response in psinI activity and what sinR is doing) is to calculate the diffusion curves for the quorum sensing response. As this paper is being written, we are conducting an experiment that will test if SinR interacts with any of the Sin AHLs; we have transformed a plasmid encoding sinR with the psinI-gfp gene and one with expR and psinI-gfp into \textit{E. coli}. Using the BioTek machine, we will add AHL and see if the fluorescence increases with any of these AHLs. This will decouple sinR and expR from the QS circuit, and we will be able to see which AHLs, if any, are the ligands for SinR and see if ExpR can bind to AHL without SinR. If our \textit{E. coli} experiments show that SinR does not interact with AHL, we will be able to confirm our suspicions that ExpR, and only ExpR, binds to the Sin AHLs.

5 Conclusion

Out of the five strains used in this experiment, only the psinI activity of MG32 responds to an increase in autoinducer concentration. The only autoinducers that induce this increased response are the 3-oxo-C\textsubscript{14}, C\textsubscript{16:1}, and 3-oxo-C\textsubscript{16:1}. ExpR appears to repress sinR expression. SinR has the biggest effect on psinI activity. These three autoinducers will be used to perform diffusion experiments once more data is collected.
6 Acknowledgements

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7 References

References


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