

## Characterization of the *Sinorhizobium meliloti* *sinR/sinI* Locus and the Production of Novel *N*-Acyl Homoserine Lactones

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*Sinorhizobium meliloti* is a soil bacterium which can establish a nitrogen-fixing symbiosis with the legume *Medicago sativa*. Recent work has identified a pair of genes, *sinR* and *sinI*, which represent a potential quorum-sensing system and are responsible for the production of *N*-acyl homoserine lactones (AHLs) in two *S. meliloti* strains, Rm1021 and Rm41. In this work, we characterize the *sinRI* locus and show that these genes are responsible for the synthesis of several long-chain AHLs ranging from 12 to 18 carbons in length. Four of these, 3-oxotetradecanoyl HL, 3-oxohexadecanoyl HL, hexadecanoyl HL, and octadecanoyl HL, have novel structures. This is the first report of AHLs having acyl chains longer than 14 carbons. We show that a disruption in *sinI* eliminates these AHLs and that a *sinR* disruption results in only basal levels of the AHLs. Moreover, the same *sinI* and *sinR* mutations also lead to a decrease in the number of pink nodules during nodulation assays, as well as a slight delay in the appearance of pink nodules, indicating a role for quorum sensing in symbiosis. We also show that *sinI* and *sinR* mutants are still capable of producing several short-chain AHLs, one of which was identified as octanoyl HL. We believe that these short-chain AHLs are evidence of a second quorum-sensing system in Rm1021, which we refer to here as the *mel* system, for “*S. meliloti*.”

Quorum sensing is a widespread phenomenon among gram-negative bacteria (for recent reviews see references 9, 16, and 33). This form of cell density-dependent gene regulation is mediated by sensing the concentrations of low-molecular-weight compounds called autoinducers, which are produced by the bacteria. A few organisms such as *Photobacterium fischeri* and *Pseudomonas aeruginosa* serve as model systems for quorum sensing. *P. fischeri*, in which autoinduction was first identified, has a relatively simple quorum-sensing system. An autoinducer synthase, LuxI, produces the *N*-(3-oxohexanoyl)-L-homoserine lactone (oxo-C<sub>6</sub>-HL), which is recognized by the LuxR regulator (11, 22, 25). LuxR then induces expression of the *lux* operon, which causes bioluminescence (47, 48). This model has recently become slightly more complicated with the identification of a second autoinducer synthase, AinS, and the regulatory protein LuxO, both of which serve to modulate quorum-sensing-induced luminescence in *P. fischeri* (25, 26, 34). On the other hand, *P. aeruginosa* has two quorum-sensing systems (*lasR/lasI* and *rhlR/rhII*) organized into a complex hierarchy, which together regulate numerous genes required for virulence (2, 36).

The most common and well-characterized gram-negative bacterial autoinducers are *N*-acyl homoserine lactones (AHLs) (see above reviews). AHLs consist of a variable acyl chain attached to a conserved homoserine lactone head group. The acyl chains can vary in length from 4 to 14 carbons. They can also vary in the nature of the substituent on the third carbon, from hydrogen to a hydroxyl or oxo group. One last charac-

teristic that can confer variability is the presence of a double bond in the acyl chain. The largest AHLs identified so far carry 14 carbon acyl chains and are synthesized by the soil bacteria *Rhizobium leguminosarum*, *Pseudomonas fluorescens*, and *Rhodobacter sphaeroides* (27, 38, 43).

The production of AHLs by several rhizobial strains was identified several years ago in survey studies (6, 46). Further research has begun to characterize quorum sensing in *Rhizobium etli* and *R. leguminosarum* and the role that it plays in the symbiotic relationship with their legume hosts. Both *R. etli* and *R. leguminosarum* were shown elsewhere to produce several AHLs, including the growth-inhibiting AHL termed bacteriocin *small* (6, 41, 43, 46). The production of *small* in both species is controlled by the *cinR* and *cinI* genes; however, the structure of the AHL is slightly different between the species. *R. leguminosarum* produces *N*-3-hydroxy-7-*cis*-tetradecanoyl-L-homoserine lactone (OH-C<sub>14:1</sub>-HL), while *R. etli* produces a partially characterized saturated long-chain 3-hydroxy-acyl homoserine lactone (8, 29, 43). In *R. leguminosarum*, *cinRI* influences the synthesis of other AHLs as well as plasmid transfer (29). For *R. etli*, *cinRI* was shown elsewhere to be expressed during symbiosis and also to be involved in bacteroid development and nitrogen fixation (8).

*Sinorhizobium meliloti* is a gram-negative soil bacterium capable of establishing a symbiotic relationship with the alfalfa plant, *Medicago sativa*. A complex sequence of signals between the plant and the bacteria is required for the successful development of nodules and subsequent nitrogen fixation (for reviews see references 3 and 49). As quorum sensing has been shown to regulate aspects of symbiosis in *R. leguminosarum* and *R. etli*, it is possible that a similar scenario exists in *S. meliloti*. To address this possibility, our lab has begun characterization of quorum sensing in two commonly studied strains

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>Sinorhizobium meliloti</i>		
Rm1021	SU47 <i>str-21</i>	28
Rm11511	Rm1021 <i>sinI</i> ::KM	This work
Rm11512	Rm1021 <i>sinR</i> ::GM	This work
<i>Agrobacterium tumefaciens</i>		
NTL4(pZLR4)	NT1 derivative carrying a <i>traG</i> :: <i>lacZ</i> reporter fusion	31
<i>Escherichia coli</i>		
MT616	MT607 (pRK600)	20
DH5 $\alpha$	See source	Life Technologies
Plasmids		
pSinR	pPCR-Script Amp SK (+) carrying the <i>sinR</i> ORF	This work
pSinR::GM	pSinR carrying <i>sinR</i> ::GM	This work
pSinRlong	pPCR-Script Amp SK (+) carrying <i>sinR</i> and surrounding region	This work
pSinRlong::GM	pSinRlong carrying <i>sinR</i> ::GM	This work
pSinIlong	pPCR-Script Amp SK (+) carrying <i>sinI</i> and surrounding region	This work
pSinIlong::KM	pSinIlong carrying <i>sinI</i> ::KM	This work
pJQ200SmSp	Shuttle vector carrying <i>sacB</i>	32
pSinIlong::KM/ <i>sacB</i>	pJQ200SmSp carrying <i>sinI</i> ::KM	This work
pSinRlong::GM/ <i>sacB</i>	pJQ200SmSp carrying <i>sinR</i> ::GM	This work

of *S. meliloti*, AK631 and Rm1021 (32). Thin-layer chromatography (TLC) analysis with the *Chromobacterium* and *Agrobacterium* indicator strains showed the presence of several AHLs in AK631 and Rm1021 culture extracts. We identified the presence of two quorum-sensing systems (the *tra* system and the *sin* system) in *S. meliloti* strain AK631 (32). The *tra* system, named for its homology to the *tra* systems in *Agrobacterium tumefaciens* and *Rhizobium* strain NGR234, resides on a large plasmid called pRme41a. This plasmid is unique to Rm41 (the AK631 parent strain) and therefore represents a quorum-sensing system that is present in AK631 but not Rm1021. The *sin* system, named for *S. meliloti*, is present in both AK631 and Rm1021 and is located on the chromosome. Since Rm1021 carries the *sin* system, but not the *tra* system, it produces only a subset of the AHLs made by AK631. In this study, we further characterize AHL production in Rm1021. We show that the *sinR/sinI* locus is responsible for the production of several novel AHLs, which have acyl side chains ranging from 12 to 18 carbons. These are the longest AHLs identified so far. Disruption of the *sinI* gene abolishes production of these long-chain AHLs, while synthesis of the other short-chain AHLs remains unaffected. We have identified one of these short-chain AHLs as octanoyl homoserine lactone (C<sub>8</sub>-HL) and propose that it and the other small AHLs are part of a second quorum-sensing system in Rm1021. Furthermore, we show that disruption of the *sin* system correlates with a delay in the appearance of nitrogen-fixing (pink) nodules, as well as an overall decrease in the number of pink nodules, indicating a role for quorum sensing in establishing a successful symbiosis with *M. sativa*.

#### MATERIALS AND METHODS

**Bacterial strains and media.** Table 1 lists the strains used in this work. All *S. meliloti* strains were cultured in Luria-Bertani broth supplemented with 2.5 mM CaCl<sub>2</sub> and 2.5 mM MgSO<sub>4</sub> (LB/MC). Antibiotics were added, as appropriate, to final concentrations of 500  $\mu$ g of streptomycin/ml, 200  $\mu$ g of neomycin/ml, 10  $\mu$ g of tetracycline/ml, and 50  $\mu$ g of gentamicin/ml. Concentrations were adjusted for

the combination of tetracycline (5  $\mu$ g/ml) and neomycin (100  $\mu$ g/ml). *A. tumefaciens* NTL4(pZLR4) (46) was grown on LB containing 50  $\mu$ g of gentamicin/ml. *Escherichia coli* strains were grown on LB containing 25  $\mu$ g of kanamycin/ml, 5  $\mu$ g of gentamicin/ml, 10  $\mu$ g of tetracycline/ml, 100  $\mu$ g of ampicillin/ml, 20  $\mu$ g of chloramphenicol/ml, or 100  $\mu$ g of spectinomycin/ml as needed. Concentrations were adjusted for the combination of tetracycline (5  $\mu$ g/ml) and kanamycin (10  $\mu$ g/ml). *S. meliloti* and *A. tumefaciens* strains were grown at 30°C, while *E. coli* was grown at 37°C.

**Construction of *sinR* and *sinI* mutants.** The *sinR* and *sinI* mutations were created by the modified PCR described in reference 19, whereby the disrupted open reading frame (ORF) is used as the primer and is incorporated into a larger template containing the wild-type copy. To generate the *sinR* disruption, the *sinR* ORF was first amplified from Rm1021 chromosomal DNA with the following primers: 5'-ATGGCTAATCAACAGGCTGTCC-3' and 5'-GATGGTGGGGA TCAGAGCATGTCC-3'. The PCR product was polished with *Pfu* DNA polymerase and then ligated into the *EcoRV* site of pPCR-Script Amp SK (+) (Stratagene), creating pSinR. The ORF was disrupted by ligating the *HindIII* fragment containing the gentamicin cassette from pUC-GM (44) into the *HindIII* site of pSinR, creating pSinR::GM. This disrupted ORF was then reamplified and used as the primer in a modified PCR described in reference 19. The template for the modified PCR was designed by amplifying a larger region of the chromosome containing the *sinR* ORF with the primers 5'-AAAGGAATTC GGCCCTCAGAATCCGCC-3' and 5'-CCGAGCAGGATCGGGAA-3' and cloning the PCR product into pPCR-Script Amp SK (+), as described above, creating pSinRlong. The PCR conditions for the modified PCR were as follows: 5 min at 95°C; followed by 18 cycles of 30 s at 95°C, 30 s at 55°C, and 6 min at 72°C; and then a final 10-min elongation step at 72°C. The PCR product was purified, and 5  $\mu$ l was transformed into DH5 $\alpha$ . This method results in the insertion of SinR::GM into pSinRlong, creating pSinRlong::GM. The *sinI* disruption was constructed by first amplifying a large region containing the *sinI* ORF with the primers 5'-CCTCGAAGAGGGGCTCG-3' and 5'-GGCGGAT CCCCTGCAGCGGCTGGTAACG-3' and cloning the PCR product into pPCR-Script Amp SK (+), as described above, creating pSinIlong, which was used as the template for the modified PCR. Next, the kanamycin cassette from Tn5 was amplified, generating ends homologous to *sinI*, by using the following primers: 5'-GGAATCGAGCCGCTTCTGCATCCAAGCACTCAGGGCGCA AGGGCTG-3' and 5'-ATGCGGATCCTGGGAGATTTCCGGGGGTGGG CGAAGAATCCAGCAT-3'. This PCR product was used as the primer along with the pSinIlong template in the modified PCR described above to generate pSinIlong::KM. Next, pJQ200SmSp was digested with *Bam*HI and filled in with Klenow fragment. The SinIlong::KM and SinRlong::GM disruptions were then amplified with *PfuTurbo*; cloned into pJQ200SmSp, creating pSinIlong::KM/*sacB* and pSinRlong::GM/*sacB*; and transformed into DH5 $\alpha$ . These constructs were used to transfer the mutations to Rm1021 via a triparental mating with MT616

as the helper strain (20). *S. meliloti* clones carrying the chromosomal disruption were selected by plating them on media containing the appropriate antibiotics and 5% sucrose (39). The resulting strains, Rm11511 and Rm11512, carry the *sinI::KM* and *sinR::GM* disruptions, respectively. An Rm1021 library was made by digesting Rm1021 chromosomal DNA with *EcoRI* and ligating it into the *EcoRI* site of pLAFR1. A complementing plasmid (p4B) carrying the *sinRI* locus was isolated by screening the library by PCR for the presence of *sinI* and *sinR*.

**Preparation and TLC analysis of crude AHL preparations.** Five-milliliter *S. meliloti* cultures were grown to saturation (optical density at 600 nm of approximately 2.4). Whole cultures (cells and supernatant) were extracted with an equal volume of dichloromethane. The extracts were dried and resuspended in 500  $\mu$ l of dichloromethane. The AHLs were analyzed by TLC and with the *A. tumefaciens* indicator strain NTL4(pZLR4) as described previously (32).

The following AHL standards were purchased from Sigma: *N*-(3-oxo-hexanoyl)-DL-homoserine lactone (oxo-C<sub>6</sub>-HL), *N*-hexanoyl-DL-homoserine lactone (C<sub>6</sub>-HL), and *N*-octanoyl-DL-homoserine lactone (C<sub>8</sub>-HL).

**In vivo labeling of AHLs.** AHLs produced by Rm1021 and its derivatives were labeled by a previously described method (12, 42). Five milliliters of supplemented minimal medium (22 g of Na<sub>2</sub>HPO<sub>4</sub>, 6 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of NaCl, 27.8 mg of CaCl<sub>2</sub>, 246 mg of MgSO<sub>4</sub>, 2 g of NH<sub>4</sub>Cl, 4 g of glucose, and 1 mg of biotin per liter) was inoculated with 50  $\mu$ l of an overnight culture and grown at 30°C. Growth was monitored by measuring the optical density at 600 nm. At approximately late log phase, 5  $\mu$ Ci of [ $\alpha$ -<sup>14</sup>C]methionine was added, and the cells were incubated with the label at 30°C for 4 h. The labeled AHLs were extracted from whole cultures with ethyl acetate. The entire extract was separated on a reverse-phase C<sub>18</sub> high-pressure liquid chromatography (HPLC) column (Varian) with a linear gradient from 0 to 95% methanol at a flow rate of 1 ml/min. One-milliliter fractions were collected, scintillation fluid (ScintiSafe Plus) was added, and each fraction was analyzed in a scintillation counter (Beckman LS 6500).

**Purification of AHLs.** A 3-liter culture of Rm1021 was grown in LB/MC to an optical density at 600 nm of 2.4 and extracted with an equal volume of dichloromethane. The extract was dried by rotary evaporation, resuspended in approximately 10 ml of dichloromethane, and dried again in a Speed Vac (Labconco). The residue was finally resuspended in 1 ml of dichloromethane. Half of this concentrated extract was separated on a reverse-phase C<sub>18</sub> column as described above, and the fractions were dried in a Speed Vac. The fractions were tested for activity with the *Agrobacterium* indicator, and the active fractions were analyzed by mass spectrometry.

**Identification of AHLs.** Electrospray ionization mass spectra (ESI MS/MS) were recorded with a Micromass (Beverly, Mass.) Quattro I triple quadrupole mass spectrometer. The source temperature was 80°C. Argon was used as the collision gas, and the collision energy was kept at 21 V for all experiments. The cone was set at 18 V, and the capillary was set at 3.88 kV. Samples were infused into the electrospray source as 75% (vol/vol) acetonitrile–25% (vol/vol) 0.22 M formic acid solutions at 4  $\mu$ l/min. For each spectrum, 20 scans at 3 s each were acquired and coadded by multiple channel analysis, and the resulting spectrum was smoothed. For all samples, the precursor ions of the mass 102 ion (P) as well as the precursor ion of the neutral loss of mass 101 (NL) were identified. The fragmentation spectra of all those precursor ions whose mass corresponded to those of acyl homoserine lactones with acyl groups that could be expected to be derived from fatty acid biosynthesis (35) were recorded as well and compared to the fragmentation spectra of the corresponding authentic compounds when these were available.

**Plant assays.** *S. meliloti* strains were incubated with *M. sativa* on standard Jensen's medium as previously described (28). Plants were checked on a weekly basis, and after 30 days plant height was measured and pink (nitrogen-fixing) and white nodules were counted. Growth conditions were 25°C, 60% relative humidity, and a 16-h light cycle.

**Growth curve analysis.** A 2-ml culture of each *S. meliloti* strain was inoculated with a single colony and grown in LB/MC in the presence of the appropriate antibiotics at 30°C until the culture was saturated. The strains were then subcultured (1:1,000) into LB/MC, and cell density was measured by monitoring the optical density at 600 nm. All growth curves were repeated at least twice.

## RESULTS

### Disruption of *sinI* abolishes production of long-chain AHLs.

Our lab has previously identified the *sinRI* locus as an AHL-producing system in Rm1021 (32). This locus corresponds to the SMC00170 and SMC00168 annotations in the *S. meliloti*

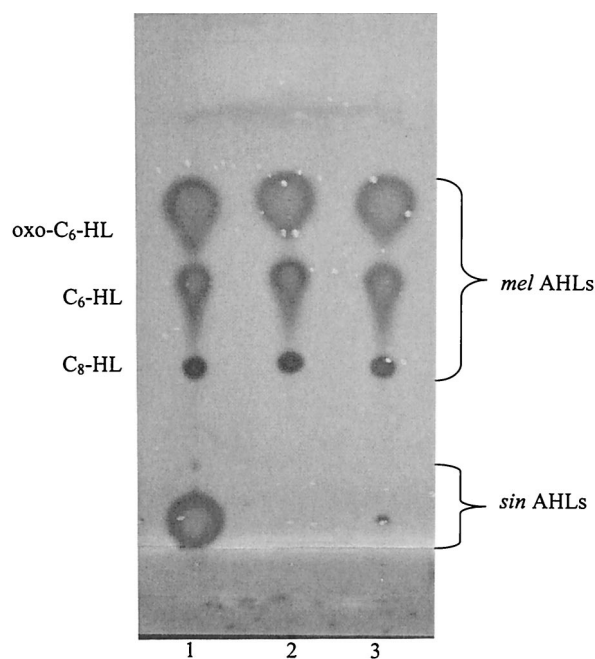


FIG. 1. Analysis of Rm1021 *sinI* and *sinR* mutations. The TLC plate was overlaid with the *A. tumefaciens* indicator. Lane 1, 500  $\mu$ l of Rm1021 extract; lane 2, 500  $\mu$ l of Rm1021 *sinI* extract; lane 3, 500  $\mu$ l of Rm1021 *sinR* extract. The mobilities of AHL standards (not shown) are indicated on the left.

Rm1021 genome database (5, 18). We showed that the *sinRI* genes, when cloned into *E. coli*, were sufficient for the production of several AHLs (32). Although not identical, the pattern of AHLs produced by *E. coli* carrying the *sinRI* genes was very similar to the pattern of AHLs made by Rm1021. Therefore, it was suggested that the *sinRI* locus was responsible for all of the AHLs made by Rm1021. In this study we wanted to further characterize the *sinRI* locus, in order to absolutely determine which AHLs were synthesized or regulated by the *sinRI* gene products. To address this issue we introduced a disruption into the SinI autoinducer synthase. Figure 1, lane 1, shows that wild-type Rm1021 produced several AHLs detected by the *Agrobacterium* indicator. A *sinI* disruption (lane 2) resulted in the dramatic loss of the AHLs which migrate close to the origin of the TLC plate. Interestingly, the pattern of the short-chain AHLs did not change. A broth-only control did not show any spots (data not shown). These results indicate that SinI is responsible only for the production of the long-chain AHLs, which remain close to the TLC origin. We will refer to these as the *sin* AHLs for clarity. In *E. coli* carrying the *sinRI* locus, we previously observed these long-chain AHLs along with several shorter-chain AHLs (32). The observation that the pattern of the shorter AHLs did not exactly match the pattern made by Rm1021, along with the result shown here that disruption of *sinI* in Rm1021 did not affect the short-chain AHLs, suggests that an additional synthase is responsible for the remaining short-chain AHLs that were detected in Rm1021 *sinI*. The additional short-chain AHLs detected in *E. coli* may be an artifact resulting from expressing *sinRI* in a nonnative background.



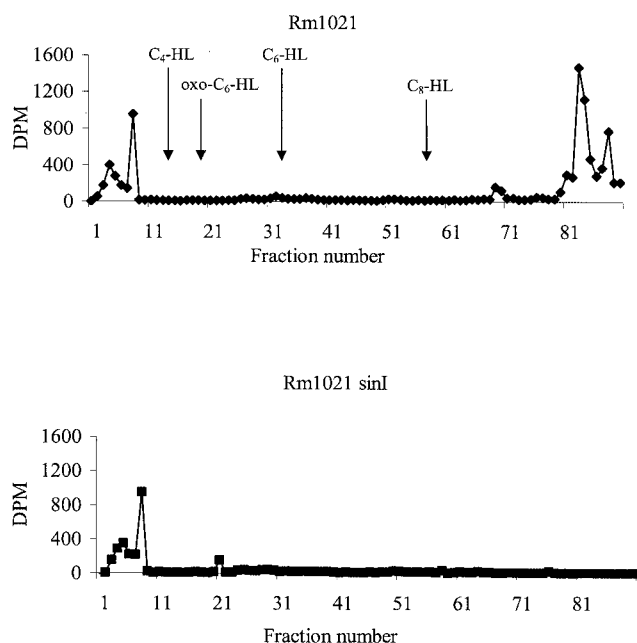


FIG. 2. Profile of labeled AHLs in Rm1021 and Rm1021 *sinI*. Five-milliliter cultures were incubated with 5  $\mu$ Ci of [ $^{14}$ C]methionine. The labeled AHLs were extracted, separated by HPLC, and then quantitated by scintillation counting. Elution times of AHL standards are indicated in parentheses: C<sub>4</sub>-HL (13 min), oxo-C<sub>6</sub>-HL (18 min), C<sub>6</sub>-HL (34 min), and C<sub>8</sub>-HL (55 min).

**SinR regulates production of the *sin* AHLs.** To establish the role of the SinR regulator in AHL production, we introduced a disruption into *sinI* and analyzed the AHL pattern (Fig. 1). As with Rm1021 *sinI*, an Rm1021 *sinR* mutant (lane 3) also had a dramatic decrease in the *sin* AHLs; however, a faint spot could be detected near the origin. This suggests that there is some residual SinI synthase activity in the *sinR* mutant. This is not surprising, since the typical quorum-sensing model begins with a basal level of expression of the AHL synthase gene, which results in the eventual accumulation of AHLs and activation of the quorum-sensing system (13, 15, 17, 45). As with Rm1021 *sinI*, the *sinR* mutant also did not affect the synthesis of the short-chain AHLs. This implies that a second quorum-sensing system exists and is not under the control of the *sin* system. For simplicity, we will refer to this new system as the *mel* system (for "*S. meliloti*"). Further evidence for the presence of the *mel* system is the fact that the *sin* mutants were still able to activate the *Agrobacterium* indicator in cross streaks on petri plates (data not shown).

**Labeling of the *sin* AHLs.** To confirm the AHL phenotypes of Rm1021 and the *sinI* mutant, we took advantage of a previously described in vivo labeling method (12, 42). This results in each newly synthesized AHL being labeled in the homoserine lactone ring. Figure 2 shows the labeled AHL profile for Rm1021. There were at least two prominent peaks at the end of the gradient (fractions 83 and 88) and an additional smaller peak at fraction 69. There were two peaks eluting at fractions 4 and 7, which correspond to unincorporated label. Interestingly, none of the short-chain AHLs seen on the TLC plate were labeled. Since the extracts analyzed by TLC were ob-

tained from cultures growing in full media, while the labeling experiments were done with minimal media, it was possible that growth conditions may have altered the AHL pattern. To address this possibility, we compared the AHL pattern of Rm1021 grown in full media to the pattern when it was grown in minimal medium. In minimal medium conditions, the *mel* AHLs were not detected by TLC analysis (data not shown), which may explain our inability to detect them in the labeling experiment.

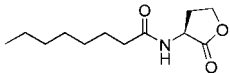
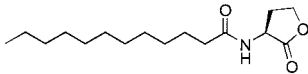
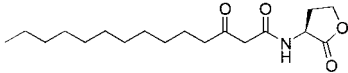
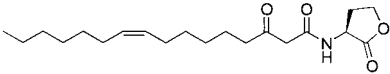
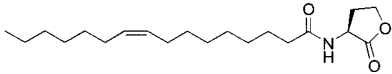
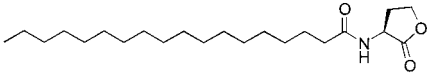
To verify that SinI is responsible for the production of the long-chain AHLs, we added [ $\alpha$ - $^{14}$ C]methionine to Rm1021 *sinI* and analyzed it as described above. Figure 2 shows that the peaks at 69, 83, and 88 are missing in a *sinI* mutant. This profile is in agreement with the TLC pattern seen in Fig. 1. It is important to note that, while the *sin* AHLs correspond to a prominent spot near the origin of the TLC plate, they are resolved into at least three prominent peaks during HPLC. This suggests that *sinI* is capable of synthesizing more than one AHL.

**Identification of AHLs produced by Rm1021.** In order to determine the identity of the *sin* AHLs, we grew a large volume of an Rm1021 culture and extracted it with dichloromethane. This extract was separated as described for the labeling experiments and then tested for activity with the *Agrobacterium* indicator. Fractions having activity were analyzed by mass spectrometry to identify their AHL structures.

The ESI MS/MS spectra of authentic AHLs showed certain consistent trends in peak height and fragmentation patterns, which aided in the identification of the AHLs produced by Rm1021. The ratio of the peak height observed for the precursor ion in the neutral loss scan (neutral loss of mass 101) to that of the precursor ion observed in the parent scan (parents of 102) was characteristic of the functionality present in the fatty acid moiety. For 2,3-unsaturated AHLs the ratio was consistently greater than 10. For unsubstituted AHLs, the ratio ranged from about 4 for short-chain acyl groups to about 0.3 for an 18-carbon chain. For 3-oxo-substituted acyl groups, the ratio was nearly 1, and for 3-hydroxy-substituted acyl groups, the ratio was considerably below 0.1. A double bond that was not conjugated to the carbonyl group seemed to have little effect on these ratios.

The fragmentation patterns of the various classes of compounds showed consistent trends as well. The 2,3-unsaturated AHLs gave a prominent peak at mass MH-101 but none at mass 102, presumably reflecting the preference towards the formation of a stable conjugated acylium ion. The unsubstituted AHLs had a small peak at mass 102 and a considerably larger one at mass MH-101. The 3-oxo-substituted AHLs showed small peaks at both mass 102 and mass MH-101. The 3-hydroxy-substituted AHLs had a peak at mass 102 and a smaller peak at mass MH-101. All compounds showed a small peak at mass MH-18, presumably representing loss of water, and one at MH-28, suggesting loss of carbon monoxide. Though none of these criteria in themselves allow the unambiguous identification of a substance giving rise to a certain peak in the ESI MS/MS, these criteria, along with the finding that the mass of the ion under question could reasonably be supposed to come from fatty acid synthesis, can allow a tentative identification, providing the means to screen crude samples quickly and with a high degree of selectivity and sensitivity.

TABLE 2. Identification of AHL structures

No.	Proposed structure	Name
1		<i>N</i> -(Tetrahydro-2-oxo-3-furanyl)-octanamide (C <sub>8</sub> -HL)
2		<i>N</i> -(Tetrahydro-2-oxo-3-furanyl)-dodecanamide (C <sub>12</sub> -HL)
3		3-Oxo- <i>N</i> -(tetrahydro-2-oxo-3-furanyl)-tetradecanamide (oxo-C <sub>14</sub> -HL)
4		3-Oxo- <i>N</i> -(tetrahydro-2-oxo-3-furanyl)-9- <i>cis</i> -hexadecenamide <sup>a</sup> (oxo-C <sub>16:1</sub> -HL)
5		<i>N</i> -(Tetrahydro-2-oxo-3-furanyl)-9- <i>cis</i> -hexadecenamide <sup>a</sup> (C <sub>16:1</sub> -HL)
6		<i>N</i> -(Tetrahydro-2-oxo-3-furanyl)-octadecanamide (C <sub>18</sub> -HL)

<sup>a</sup> See Results.

Comparison with an authentic compound then gives a definite identification.

Various novel AHLs were identified in the Rm1021 HPLC fractions (Table 2). Among them is oxo-C<sub>14</sub>-HL, C<sub>16:1</sub>-HL (Fig. 3) (also reported previously by M. P. Johnston, A. Eberhard, S. Gallacher, and L. A. Glover [submitted for publication]), oxo-C<sub>16:1</sub>-HL (Fig. 4), and C<sub>18</sub>-HL. Comparison of the natural-sample spectra with those of authentic samples supports our assignments in all cases. In the cases of oxo-C<sub>14</sub>-HL and oxo-C<sub>16:1</sub>-HL, an authentic standard was not available, so the spectra were compared to those of synthetic oxo-C<sub>12</sub>-HL and oxo-C<sub>18:1</sub>-HL, respectively. The smallest AHL that was identified was octanoyl-HL (C<sub>8</sub>-HL). Accordingly, we detected a spot on the TLC plate with mobility similar to that of the C<sub>8</sub>-HL standard (Fig. 1).

**Analysis of *sin* mutants in free-living conditions.** Previous research has shown that the production of OH-C<sub>14:1</sub>-HL by *R. leguminosarum*, as well as production of the uncharacterized saturated long-chain AHL by *R. etli*, plays a role in regulating growth (8, 43). These particular AHLs were shown to induce stationary phase and to contribute to survival in stationary phase. Since the *sin* locus makes several long-chain AHLs, including one with a 14-carbon acyl chain, we were interested to see if disrupting *sinI* would have an effect on the growth rate. We measured the growth of Rm1021, Rm1021 *sinI*, and Rm1021 *sinR* in LB/MC (Fig. 5). It is clear that neither of the mutations had an effect on growth in these conditions, since the growth curves were identical to that of wild-type Rm1021. Similar results were obtained with mannitol-glutamate minimal media (data not shown). This suggests that under these conditions the *sin* AHLs do not seem to play a role in regulating growth phase in Rm1021.

**Effect of *sinI* and *sinR* disruptions on symbiosis.** Studies of *R. leguminosarum* indicate that quorum sensing plays a role in symbiosis, such as controlling nodulation efficiency (7). To determine whether the *sin* system also has a role in symbiosis, we inoculated the *sinI* and *sinR* mutants onto *M. sativa* (alfalfa). Figure 6 shows the final results after incubating the plants for 1 month. While neither of the mutants had a Fix<sup>-</sup> phenotype, we did observe a slight, but consistent, decrease in the average number of pink nodules for both Rm1021 *sinI* and Rm1021 *sinR*. In addition, we monitored the progress of nodulation by checking the plants on a weekly basis. Interestingly, we found that the *sinI* and *sinR* mutants had a slight delay in the appearance of nodules (data not shown). After 1 week, plants inoculated with Rm1021 had developed numerous white nodules and a few pink nodules. Plants inoculated with the *sinI* and *sinR* mutants had few, if any, white nodules, and there were no pink nodules present. By the second week all plants had developed pink nodules; however, there were fewer pink nodules on plants inoculated with the *sin* mutants than on those inoculated with Rm1021. This suggests that the *sinRI* locus may regulate one or more genes involved in establishing a successful symbiotic relationship. Clearly, the role of the *sin* system, and the possible target genes of SinR, needs to be investigated to further determine the nature of quorum-sensing regulation in symbiosis.

## DISCUSSION

Recent work in our lab identified the *sinRI* locus as an AHL-producing system common to *S. meliloti* strains AK631 and Rm1021 (32). We show here that the *sinI* and *sinR* genes

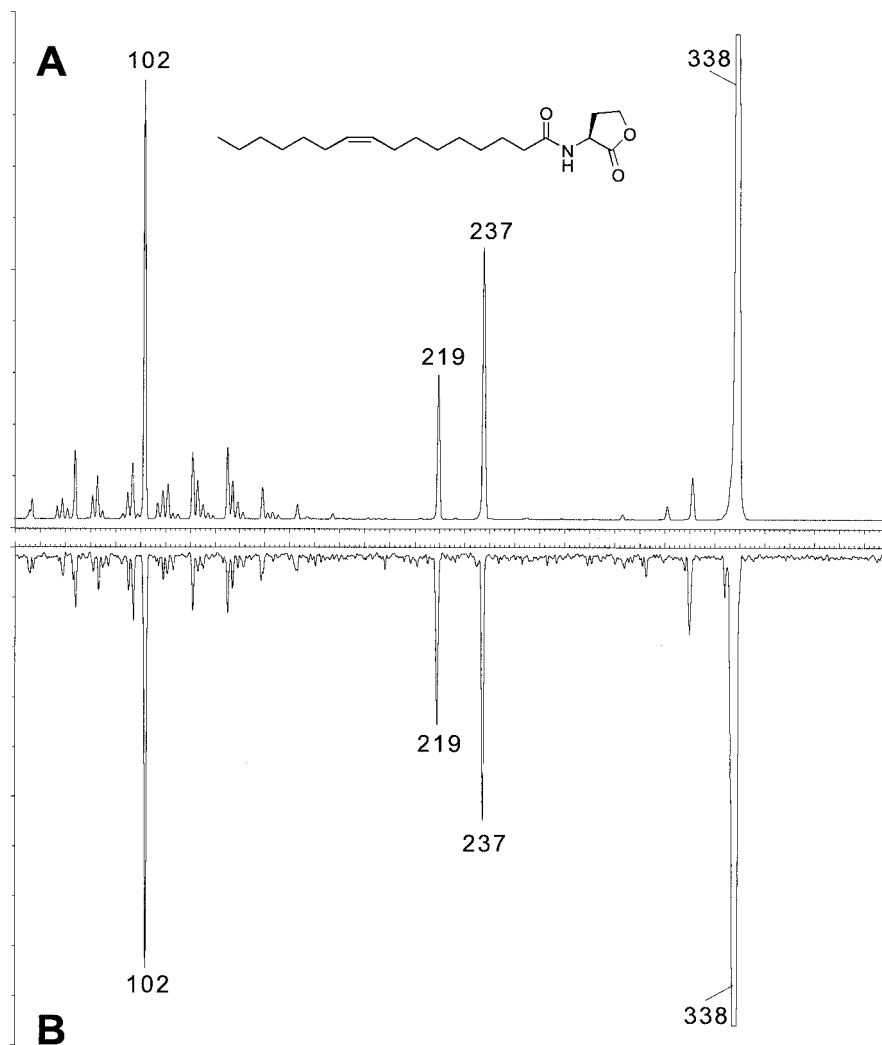


FIG. 3. Comparison of the collisionally induced dissociation spectrum for the precursor ion observed at  $m/z = 338$  in the natural sample to that of an authentic sample. (A) Synthetic *N*-(tetrahydro-2-oxo-3-furanyl)-9-*cis*-hexadecenamide recorded for precursor ion  $m/z = 338$ . (B) Same scan carried out on the semipurified natural material.

are responsible for the production of several long-chain AHLs. When the autoinducer synthase *sinI* was disrupted, the long-chain AHLs were no longer present (Fig. 1 and 2). Likewise, disrupting the regulator, *sinR*, resulted in a dramatic decrease in the long-chain AHLs (Fig. 1). This is consistent with the role of SinR as a positive regulator for the *sinI* gene. The fact that there is some residual AHL synthesis suggests that there is a basal level of expression of the *sinI* gene even in the absence of SinR. This phenomenon has been observed before and is the basis for the quorum-sensing mechanism (13, 15, 17, 45). These results strongly suggest that the *sinRI* locus is responsible, either directly or indirectly, for the production of the long-chain AHLs. It remains a possibility that the phenotypes observed in the *sin* mutants may be an indirect effect.

To further characterize the AHLs made by the *sinRI* locus, we conducted an *in vivo* labeling experiment, which showed that multiple long-chain AHLs could be detected from Rm1021 cultures (Fig. 2). Subsequent mass spectrometry analysis de-

termined the structures of these AHLs to be *N*-(tetrahydro-2-oxo-3-furanyl)-dodecanamide ( $C_{12}$ -HL), 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-tetradecanamide (oxo- $C_{14}$ -HL), 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-9-*cis*-hexadecenamide (oxo- $C_{16:1}$ -HL), *N*-(tetrahydro-2-oxo-3-furanyl)-9-*cis*-hexadecenamide ( $C_{16:1}$ -HL), and *N*-(tetrahydro-2-oxo-3-furanyl)-octadecanamide ( $C_{18}$ -HL) (Table 2). To the best of our knowledge, this is the first report of naturally occurring oxo- $C_{14}$ -HL, oxo- $C_{16:1}$ -HL, and  $C_{18}$ -HL. The  $C_{16:1}$ -HL has also been observed in an uncharacterized marine bacterium designated 667-2 (Johnston et al., submitted). These novel AHLs are the longest ones identified to date. There may still be additional *sin* AHLs, which we have not been able to identify, but these will likely be minor species. Other organisms, such as *R. leguminosarum*, *R. sphaeroides*, and *P. fluorescens*, were shown elsewhere to produce AHLs with acyl chains that were 14 carbons in length (27, 38, 43). However, in those studies, only the culture supernatants were analyzed. Since the long-chain AHLs are highly

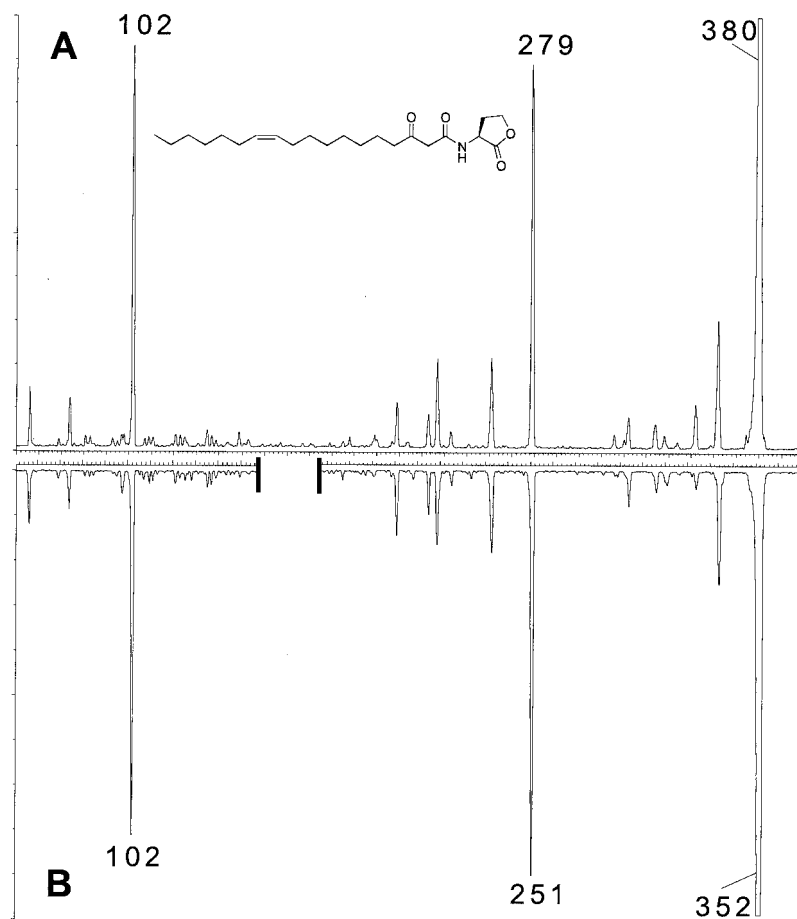


FIG. 4. Comparison of the collisionally induced dissociation spectrum for the precursor ion observed at  $m/z = 352$  in the natural sample to that of an analogous authentic sample (3-oxo- $C_{18:1}$ -HL). (A) Synthetic 3-oxo- $N$ -(tetrahydro-2-oxo-3-furanyl)-9-*cis*-octadecenamide for precursor ion  $m/z = 380$ . (B) Semipurified natural material. To allow ready visualization of the correspondence, the figure was broken by 28 mass units to account for the two extra carbon atoms that the 3-oxo- $C_{18:1}$ -HL authentic sample has in the region of the spectrum that involved fragmentation of the acyl chain.

hydrophobic, it is likely that they will partition to the cell membrane. Therefore, we reasoned that by extracting whole cultures (cells and supernatant) we would increase the recovery of long-chain AHLs. It is possible that AHLs with acyl chains longer than 14 carbons may be present in other organisms as well, and extracting whole cultures may make their recovery and detection more efficient.

In addition to the long-chain AHLs produced by the *sinRI* locus, Rm1021 makes several short-chain AHLs (Fig. 1). One of these AHLs was identified as  $N$ -(tetrahydro-2-oxo-3-furanyl)-octanamide ( $C_8$ -HL). These AHLs seem to represent a quorum-sensing system separate from the *sinRI* system, since mutations in *sinI* and *sinR* had no effect on the levels of the remaining short-chain AHLs (Fig. 1). Although we have not identified the genes responsible for the synthesis of these AHLs, we have tentatively named this additional system the *mel* system (*S. meliloti*) for convenience. A search of the Rm1021 genome database did not reveal any other LuxI-type or LuxM-type AHL synthases. However, the genome does contain an ORF designated as a putative 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (SMc00714) (5, 18). Recent work by

Laue et al. showed that a homologous gene, termed *hdtS*, was responsible for the production of multiple AHLs in *P. fluorescens* (27). Since *hdtS* could direct AHL synthesis but was not homologous to either *luxI* or *luxM*, it was proposed that it represented a new class of AHL synthase. It is possible that the *S. meliloti* homolog (SMc00714) is a member of the HdtS family and is responsible for the synthesis of  $C_8$ -HL and the other short-chain AHLs (25).

It is interesting that the length and variety of the acyl chains in the AHLs produced as a result of the SinI synthase activity. Clearly SinI is quite promiscuous in its ability to interact with the proper acyl carrier protein (ACP). ACPs are involved not only in the synthesis of fatty acyl chains but also in their transfer during phospholipid, lipid A,  $\alpha$ -hemolysin (23, 24), and nodulation (Nod) factor biosynthesis (10, 30). In *E. coli*, all of these functions seem to be realized by a single and absolutely essential ACP, which is produced constitutively from the *acpP* gene (24, 40). In addition to AcpP, *S. meliloti* potentially encodes four additional ACPs. One of them is the gene product of *nodF*, which is involved, together with NodE, in the synthesis of polyunsaturated fatty acids that are host-specific

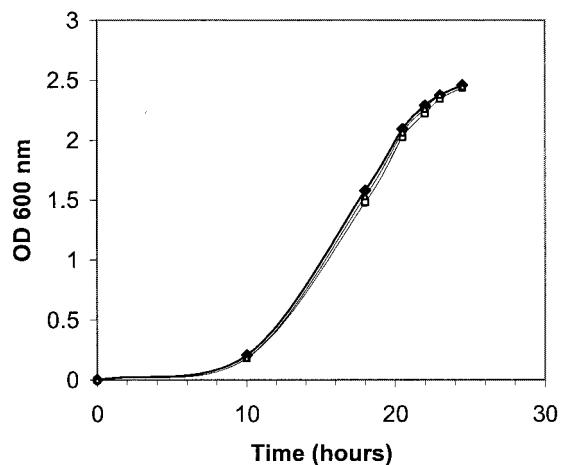


FIG. 5. Effect of *sinI* and *sinR* disruptions on growth. All strains were grown in LB/MC, and growth was monitored by measuring the optical density at 600 nm. Symbols: solid diamonds, Rm1021; open squares, Rm1021 *sinI*; open triangles, Rm1021 *sinR*.

substituents of Nod factors (50). The *rkpF* gene product in *S. meliloti* 41 is a novel ACP, which presumably functions, in combination with other gene products, in the synthesis of an unusual fatty acid or  $\beta$ -ketide (14, 37). An additional novel ACP, named AcpXL, seems to be involved in the transfer of the unusually long 27-hydroxyoctacosanoic acid to a sugar backbone during lipid A biosynthesis in *R. leguminosarum* (4). Finally, a fifth putative ACP is encoded by the SMC01553 locus in the *S. meliloti* genome (5, 18). From our work it seems that SinI produces mostly long-chain AHLs, while the putative MelI synthase is involved in the synthesis of mostly short-chain AHLs. It is possible that the SinI and MelI synthases could interact specifically with one or more of the specialized ACPs in *S. meliloti*. It would be interesting to determine if mutations in any of the putative ACP genes in *S. meliloti* (*nodF*, SMC01553, *acpXL*, or *rkpF* in Rm41) affect the biosynthesis of AHLs. Alternatively, the *sinRI* locus might regulate another

synthase gene, which would direct the synthesis of one or more of the long-chain AHLs.

In addition to characterizing the *sinRI* locus, we are also interested in identifying potential downstream target genes which are regulated by quorum sensing. In *R. leguminosarum*, the long-chain AHL, OH-C<sub>14:1</sub>-HL, not only induces early onset of stationary phase but also influences nodulation efficiency (7, 21). Among the several long-chain AHLs made by *S. meliloti* is an oxo-C<sub>14</sub>-HL, which is similar to the OH-C<sub>14:1</sub>-HL in *R. leguminosarum*. However, disruption of *sinI*, which abolishes production of oxo-C<sub>14</sub>-HL, did not alter the dynamics of the growth curve (Fig. 5). Therefore, the long-chain AHLs do not seem to regulate growth phase in Rm1021. It should be noted, however, that the presence of the symbiotic plasmid pRL1JI is required for growth sensitivity to OH-C<sub>14:1</sub>-HL (1); therefore, we cannot rule out the possibility that the *sinRI* locus or the *sin* AHLs may regulate growth phase in other genetic backgrounds, such as Rm41. Since quorum sensing has also been shown to control aspects of symbiosis in other organisms, we decided to study the effects of the *sinI* and *sinR* disruptions on nodulation of the alfalfa plant, *M. sativa*. Interestingly, the *sin* mutants showed a slight decrease in the number of nitrogen-fixing (pink) nodules per plant, as well as a delay in the appearance of the pink nodules. These results indicate that, although nodulation is not abolished in the mutants, the process is definitely impaired. A similar phenomenon was observed for *R. leguminosarum*, where it was shown that the *rhiABC* operon is controlled by quorum sensing and, in turn, regulates nodulation efficiency (7, 21). Similarly, in *R. etli* the *rai* quorum-sensing system regulates the number of nodules, while the *cin* system controls symbiosome development and nitrogen fixation (8, 41). While we do not know which genes are specifically controlled by the *S. meliloti* *sin* system, our work suggests that one or more target genes are involved in establishing a successful symbiotic relationship. Further work will need to be done to identify these target genes. With the power of proteomics, it should be possible to identify genes that are expressed differently in the *sin* mutants compared to

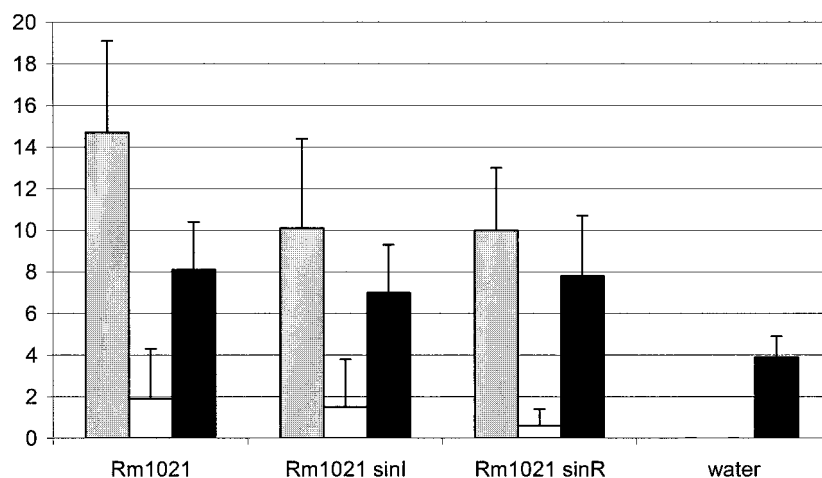


FIG. 6. Effect of *sinI* and *sinR* disruptions on alfalfa nodulation. Alfalfa plants were inoculated with Rm1021, Rm1021 *sinI*, and Rm1021 *sinR*. Gray bars, number of pink nodules; white bars, number of white nodules; black bars, plant height (centimeters). Data represent scoring of 20 plants per *S. meliloti* strain after 30 days of incubation.



those in the wild type. Characterization of those genes should provide valuable insights into the role of quorum sensing in symbiosis.

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