THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 1, Issue of January 4, pp. 462–468, 2002

Printed in U.S.A.

The *cin* Quorum Sensing Locus of *Rhizobium etli* CNPAF512 Affects Growth and Symbiotic Nitrogen Fixation*

Received for publication, July 16, 2001, and in revised form, October 18, 2001
Published, JBC Papers in Press, October 24, 2001, DOI 10.1074/jbc.M106655200

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***Rhizobium etli* CNPAF512 produces an autoinducer that inhibits growth of *Rhizobium leguminosarum* bv. *viciae* 248 and activates the *Agrobacterium tumefaciens tra* reporter system. Production of this compound in *R. etli* is dependent on two genes, named *cinR* and *cinI*, postulated to code for a transcriptional regulator and an autoinducer synthase, respectively. NMR analysis of the purified molecule indicates that the *R. etli* autoinducer produced by CinI is a saturated long chain 3-hydroxy-acyl-homoserine lactone, abbreviated as 3OH-(slc)-HSL. Using *cin-gusA* fusions, expression of *cinI* and *cinR* was shown to be growth phase-dependent. Deletion analysis of the *cinI* promoter region indicates that a regulatory element negatively controls *cinI* expression. Mutational analysis revealed that expression of the *cinI* gene is positively regulated by the CinR/3OH-(slc)-HSL complex. Besides 3OH-(slc)-HSL, *R. etli* produces at least six other autoinducer molecules, for which the structures have not yet been revealed, and of which the synthesis requires the previously identified *raiI* and *raiR* genes. At least three different autoinducers, including a compound co-migrating with 3OH-(slc)-HSL, are produced in *R. etli* bacteroids isolated from bean nodules. This is further substantiated by the observation that *cinI* and *cinR* are both expressed under symbiotic conditions. Acetylene reduction activity of nodules induced by the *cin* mutants was reduced with 60–70% compared with wild-type nodules, indicating that the *R. etli* 3OH-(slc)-HSL is involved in the symbiotic process. This was further confirmed by transmission electron microscopy of nodules induced by the wild type and the *cinI* mutant. Symbiosomes carrying *cinI* mutant bacteroids did not fully differentiate compared with wild-type symbiosomes. Finally, it was observed that the *cinR* gene and *raiR* control growth of *R. etli*.**

Although bacteria are unicellular organisms, they often show group behavior. For this, bacteria have to monitor their own population size. This can be achieved by means of autoinduction. Cell-cell communication using *N*-acyl-homoserine lactone (AHL)[1] signals is one of the few known mechanisms through which bacteria can communicate with each other and is a widespread phenomenon in Gram-negative bacteria (1, 2), including plant-associated bacteria (3, 4). AHLs mainly vary with respect to the length (4–14 carbons) and the substituent (H, O, or OH) at the third carbon of the acyl side chain. The AHL signal is released into the environment, either by passive diffusion, as observed for 3O-C_6-HSL in *Vibrio fischeri* and *Escherichia coli* cells (5) or by a combination of diffusion and active efflux in *Pseudomonas aeruginosa* (6) and accumulates with growth of the bacterial population. At least in *V. fischeri*, the signal freely diffuses back into the cells such that its intracellular concentration also rises as a function of the increase in bacterial population. Transduction of this information to response regulators of gene expression leads to the elaboration of an appropriate phenotype at high cell densities.

Using the *Agrobacterium tumefaciens tra* reporter system to detect autoinducer molecules, members of the genus *Rhizobium* showed the greatest diversity, with some producing as few as one and others producing as many as seven detectable signals (7). In *Rhizobium leguminosarum* bv. *viciae*, the *cin* locus encodes a master regulatory system. Mutation of *cinIR* abolishes the production of *N*-(3*R*)-hydroxy-7-*cis*-tetradecenoyl-L-homoserine lactone (3OH-$C_{14:1}$-HSL), also termed "small", and reduces the synthesis of AHLs produced by the enzymes encoded by *raiI*, *traI*-like, or *rhiI* (8). The reduced levels of C_6-HSL and C_8-HSL and decreased *rhiR* expression cause a repression of the rhizosphere-expressed genes in *cinI* or *cinR* mutants (8–10). Furthermore, 3OH-$C_{14:1}$-HSL induces the stationary phase (9) whereas mutation of *cinI* has little effect on growth or nodulation of the host plant (8, 11).

Rhizobium etli CNPAF512, a nitrogen-fixing symbiont of *Phaseolus vulgaris*, produces at least seven different autoinducer molecules (12). Rosemeyer *et al.* (12) identified the *raiIR* quorum-sensing system in *R. etli*. Examination of different *rai* mutants for nodulation of beans showed that *raiI* is involved in the restriction of nodule number, whereas nitrogen-fixing activity per nodule is not affected. The culture supernatant of a *raiI* mutant revealed only three different autoinducer molecules. One of them induces a growth-inhibitory effect on *R. leguminosarum* bv. *viciae* 248, similar to the low molecular

* Parts of this study were presented at the American Society for Microbiology Conference on Cell-Cell Communication in Bacteria, July 6–9, 2001, Snowbird, UT. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF393621.

§ Recipient of a fellowship from the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie (IWT).

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[1] The abbreviations used are: AHL, *N*-acyl-homoserine lactone; TEM, transmission electron microscopic analysis; ORF, open-reading frame; HPLC, high-performance liquid chromatography; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ARA, acetylene reduction activity; TLC, thin layer chromatography; DKP, diketopiperasines.

weight bacteriocin "small", which is common in fast-growing rhizobia. The properties, growth inhibition, and autoinducer activity, are features reported for 3OH-$C_{14:1}$-HSL, produced by *R. leguminosarum* bv. *viciae* (13).

Here we report on the *cin* locus, the second quorum-sensing system in *R. etli* CNPAF512 that is expressed under both free-living and symbiotic conditions and is involved in the production of a 3OH-(slc)-HSL (slc, saturated long chain). Despite high sequence conservation, the *cin* locus of *R. etli* and *R. leguminosarum* bv. *viciae* appear to control different functions. Mutational analysis of *R. etli* revealed that the *cin* system regulates growth and fulfills a key role in bacteroid differentiation and nitrogen fixation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—E. coli was grown in Luria-Bertani (LB) medium at 37 °C (14). *Rhizobium* was grown at 30 °C in TY or AMS medium (15). *A. tumefaciens* NT1 was grown in AB medium at 28 °C (16). Antibiotics were added as appropriate. To study bacterial growth over a long period of time, overnight cultures of the strains were diluted in 10 mM $MgSO_4$ to an absorbance at 595 nm (A_{595}) of 0.3 (dilution ~10-fold). Subsequently, these cultures were diluted 10-fold after which 295 μl of growth medium was inoculated with 5 μl of bacterial suspension (total dilution ~6000-fold). Bacteria were grown, and the absorbance was measured automatically each 30 min during at least 6 days in a BioscreenC (Labsystems Oy). For each time point, the average optical density was calculated from five independent measurements.

Plant Experiments, Bacteroid Isolation, and Preparation of Bacteroid-free Supernatant—Phaseolus vulgaris cv. Limburgse vroege seedlings were planted in Snoeck medium, which is optimized for *in vitro* growth of common bean.[2] Plants were inoculated and grown essentially as described by Michiels *et al*. (17). Acetylene reduction activity was determined 3 weeks after inoculation. For expression analysis during symbiosis, bacteroids were purified from plant material by differential centrifugation (17). This protocol was slightly adapted for the extraction of bacteroid autoinducers. Nodules from 1–2 plants (± 1.5 g) were collected in a Falcon tube (15 ml) containing 0.2 g of polyvinyl polypyrolidone, and magnesium phosphate buffer was added to a final volume of 6 ml and subsequently crushed. Next, the plant material was removed by differential centrifugation. To lyse the bacteria, the suspension was supplemented with SDS (final concentration, 1%) and proteinase K (final concentration, 100 μg/ml) and incubated for 1 h at 37 °C. After incubation, the cell debris was removed by centrifugation at 6000 rpm after which the cell-free supernatant was immediately extracted to isolate the autoinducers.

Qualitative and quantitative analysis of β-glucuronidase (GusA) activity was performed as described elsewhere (17, 18). For transmission electron microscopic analysis (TEM), thin sections of 3-week-old nodules were prepared as described by Xi *et al*. (19), and analyzed in a Zeiss EM 900 electron microscope.

Extraction and Detection of Autoinducers—Rhizobium strains and *A. tumefaciens* transformants were tested for activation of the *A. tumefaciens tra* reporter system and for bacteriostatic activity toward the sensitive strain *R. leguminosarum* bv. *viciae* 248 as described by Schripsema *et al*. (13). To extract autoinducers, strains were first grown to the stationary phase. Cell-free supernatant from either a free-living liquid culture or from symbiotic bacteroids (see above) was extracted, and the autoinducers were detected on TLC by the *tra* reporter system as described by Rosemeyer *et al*. (12).

Isolation of the R. etli 3OH-(slc)-HSL—Ethyl acetate extracts from 10 liters of stationary phase cultures of FAJ4010 were reconstituted in 50% acetonitrile in water and subjected to solid-phase extraction using Waters OASIS HLB cartridges. Fractions were eluted with an increasing concentration of methanol in water (50–100%, v/v). Positive fractions on the *A. tumefaciens tra* reporter and *R. leguminosarum* bv. *viciae* 248 growth inhibition assays were collected, dried, and redissolved in 50% acetonitrile in water and applied to a C18 Phenomenex Bondclone HPLC column. Fractions were eluted with a linear gradient of acetonitrile in water (40–100%, v/v) over a 30-min period at a flow rate of 1 ml/min and monitored at 200 nm. Positive fractions were re-chromatographed using an isocratic mobile phase (50% acetonitrile in water; 1 ml/min), and the active subfraction was analyzed by NMR spectroscopy.

[2] C. Snoeck, unpublished data.

DNA Techniques and Nucleotide Sequencing—Standard techniques were used for DNA manipulations (14). Restriction enzymes were used according to the manufacturer's instructions. DNA probes for Southern hybridization were labeled with digoxigenin. An ordered series of sequencing clones was obtained via restriction enzyme mapping of pFAJ4003 and *Exo*III deletion procedures (Erase-A-Base® Promega). Nucleotide sequencing of *cinR*, *cinI,* and the flanking regions was accomplished by using the A.L.F. sequencer (Amersham Biosciences).

Cloning of the cinR-cinI Gene Region and Construction of Mutants—The 5.3-kb *Eco*RI fragment from pFAJ4000 containing *cinR*, *cinI*, *orf123,* and *orf140* was first cloned into pBluescriptIIKs$^+$ yielding pFAJ4003. A *Pst*I-*Eco*RI fragment of pFAJ4003, lacking the 5′-end of *orf140*, was *Eco*RI-*Pst*I subcloned in the broad host range vector pLAFR3 (pFAJ4012; Fig. 1). Furthermore, pFAJ4013 was made by insertion of the 5.3-kb *Eco*RI fragment from pFAJ4003 in pPZP200, in which part of the multiple cloning site between *Xho*I and *Pac*I was deleted.

A fragment containing *cinR* and *cinI* was amplified via PCR using primers Rhi15 (5′-ATGGGAATTCATCCAGTGCCGAGGAGATAC-3′) and Rhi16 (5′-TAGAGGATCCTCGGCATCATCATCACCTCG-3′) and pFAJ4003 as template DNA. The resulting 3-kb fragment was digested with *Eco*RI and *Bam*HI and cloned in pUCNotI-ΔS (pUCNotI derivative lacking the *Sph*I recognition site via restriction digest and blunt ligation) yielding plasmid pFAJ4004 (Fig. 1). The 2.2-kb *Bam*HI fragment from pHP45Ω-Km containing a Km^r cassette was ligated into the unique *Sph*I site of *cinI* in pFAJ4004 after blunting of the fragments. The *cinRcinI*::Km locus was further cloned into the *sacB* suicide vector pJQ200uc1 as a 5.2-kb *Not*I fragment to obtain pFAJ4006. The nonpolar *cinR* point mutation and an additional frameshift were introduced via the QuickChange™ site-directed mutagenesis (Stratagene) using primers Rhi32 (5′-CTATGCCATGCTGGTACCATGCCCAGAAGCACG-3′) and Rhi33 (5′-CGTGCTTCTGGGCATGGTACCAGCATGGCATAG-3′) on pFAJ4004. As a result of the mutation, a new and unique *Kpn*I site (GGT**ACC**; mutations are shown in bold: insertion of A; substitution G with C) was created in *cinR*. Subsequently, the 3-kb *Not*I fragment containing the mutated *cinR* gene was inserted into pJQ200uc1 creating pFAJ4007. The *cinR*::Sp mutation was made by introducing the 2-kb spectinomycin resistance cartridge from pHP45Ω into the unique *Kpn*I site within *cinR* of pFAJ4007 after blunting of the fragments, resulting in pFAJ4009. The plasmids pFAJ4006, pFAJ4007, and pFAJ4009 were introduced into *R. etli* CNPAF512, and double recombinants were selected as described previously (12) creating a *cinI* mutant (FAJ4006) and two *cinR* mutants (FAJ4007, FAJ4009; Fig. 1). A cartridge containing the promoterless *gusA* gene and a spectinomycin resistance gene from pWM5 was removed with *Sma*I and ligated into the *Xho*I site of pFAJ1327 (containing *raiI*, *raiR*, and *orf1*) after blunting of the vector, yielding plasmid pFAJ4010. For the construction of a *R. etli raiI* mutant strain, pFAJ4010 was introduced into the *R. etli* CNPAF512 wild-type strain, yielding FAJ4010. Tri-parental conjugation of pFAJ4006 (*cinI*::Km) into FAJ4010 resulted in the *raiI*::Sp *cinI*::Km double mutant FAJ4013.

Construction of Fusions—Plasmid-borne P_{cinR}-*gusA* and P_{cinI}-*gusA* fusions were constructed in the promoter probe vector pFAJ1703 (20). The *cinR* promoter region was amplified by PCR with FAJ1337 as a template using primers Rhi15 and Tn*5*-B (5′-GGTTCCGTTCAG-GACGCTAC-3′). The resulting 1.2-kb fragment was cloned into the *Hpa*I site of pFAJ1703 after blunting of the fragments, yielding plasmid pFAJ4011. To construct pFAJ4014 (*cinI-gusA* fusion carrying a mutant *cinR* gene region), the 1.8-kb *Eco*RI-*Sph*I fragment of pFAJ4007 was blunt-end ligated into the *Hpa*I site of pFAJ1703. pFAJ4015 is a *Kpn*I deletion derivative of the *cinI-gusA* fusion pFAJ4014 (Fig. 1).

RESULTS

Identification of the cin Locus of R. etli CNPAF512—A Tn*5*-induced mutant library of *R. etli* CNPAF512 was screened using the growth inhibition assay (13). The mutant FAJ1337 no longer inhibited growth of *R. leguminosarum* bv. *viciae* 248. By means of inverse PCR on *Eco*RI-digested genomic DNA of FAJ1337 with transposon-specific primers, ~10 kb of the transposon flanking DNA of FAJ1337 was amplified and cloned. Subsequently, *Pst*I fragments of the amplified DNA were subcloned into pUC18 and tested for autoinducer production in *E. coli* using the *tra* system of *A. tumefaciens* as a reporter (7). One positive clone was obtained, and the corresponding 7.5-kb *Pst*I fragment, carrying part of the cloning vector, was subsequently used as a probe in a Southern hybrid-

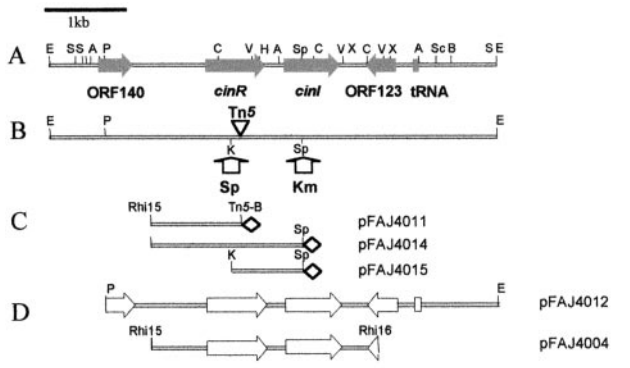

FIG. 1. **Gene organization of the *cin* locus in *R. etli* and construction of *gusA* fusions and mutant strains.** *Panel A*, physical and genetic map of the 5.3-kb fragment containing the *cin* locus from *R. etli* CNPAF512. *Arrows* indicate directions of transcription. Restriction sites: *A*, *Apa*I; *B*, *Bam*HI; *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *S*, *Sal*I; *Sc*, *Sac*I; *Sp*, *Sph*I; *V*, *Eco*RV; *X*, *Xho*I. *Panel B*, *cinR* and *cinI* insertional mutants: *cinR*::Tn*5* (FAJ1337); a non-polar point mutation in *cinR* (FAJ4007); *cinR*:: Sp^r (FAJ4009); *cinI*:: Km^r (FAJ4006). *Panel C*, construction of *cinR-gusA* (pFAJ4011) and *cinI-gusA* fusions (pFAJ4014, pFAJ4015). The position of the *gusA* gene is indicated. *Panel D*, construction of pFAJ4012 and pFAJ4004. Primers and restriction sites used for construction are indicated.

ization of a *R. etli* CNPAF512 genomic library, constructed in pLAFR1 (21). A 5.3-kb *Eco*RI fragment from clone pFAJ4000 was found to give a positive hybridization signal. FAJ1337 was complemented for growth inhibition of the sensitive strain *R. leguminosarum* bv. *viciae* 248 by both pFAJ4000 and pFAJ4012 (Fig. 1).

DNA Sequence Analysis of the R. etli CNPAF512 cin Locus—DNA sequence analysis of the cloned 5.3-kb *Eco*RI fragment revealed five complete open reading frames (ORFs) as illustrated in Fig. 1. The ORFs were identified and the start codon was assigned on the basis of the GC content (22), the preferential codon usage (searchcutg, GCG-package Wisconsin), and similarity with known genes.

One ORF (726 bp) codes for a putative protein of 241 amino acids with a calculated molecular mass of 27.3 kDa. The putative protein is similar to several LuxR-type transcriptional activators such as CinR (96% amino acid identity) of *R. leguminosarum* bv. *viciae* (AAF89989), CerR (30% identity) of *Rhodobacter sphaeroides* (AAC46021) and RaiR (31% identity) of *R. etli* (AAC38173). Because of the high amino acid identity of the *R. etli* putative protein with CinR, it was given the same name. Alignment of *R. etli* CinR with *E. coli* NarL (23), designates a helix-turn-helix motif between residues 196 and 220. *In silico* analysis of the *cinR* non-coding region revealed a putative terminator (nucleotides 2615–2650, $\Delta G = -26.3$). PCR analysis with a Tn*5*-specific primer combined with primers within the coding sequence of *cinR* or *cinI* (see below) indicated that the transposon in FAJ1337 is inserted between nucleotides 423 and 424 of *cinR*.

A second ORF of 666 bp, which is unidirectional with *cinR*, is found 224-bp downstream of *cinR*. While the deduced amino acid sequence is most related to CinI (95% identity) of *R. leguminosarum* bv. *viciae* (AAF89990), it is also similar to CerI (33% identity) of *R. sphaeroides* (AAC46022) and RaiI of *R. etli* (39% identity) (AAC38172). The putative protein with a calculated molecular mass of 25.0 kDa was named CinI. CinI contains 10 invariant amino acids typical for the LuxI family of autoinducer synthases (24) of which seven (R24, E43, D45, D48, R70, E101, R104; numbered with respect to *R. etli* CinI) may take part in the *S*-adenosyl-methionine binding site (25). In the intergenic region between *cinI* and ORF123 (see below), two putative terminators (nucleotides 3520–3576, $\Delta G = -23.8$; nucleotide 3694–3735, $\Delta G = -32.4$) were found.

Immediately downstream of *cinI*, a short ORF123 (368 bp), located on the opposite strand, encodes a putative response regulator of the CheY family with 94% identity to the CheY like protein of *R. leguminosarum* bv. *viciae* (AAF89991), 40% identity to a probable response regulator of *Mesorhizobium loti* (BAB49462) and 35% identity to the FixL receiver domain of *R. etli* (AAG00949). The *R. etli* response regulator encoded by ORF123 contains the conserved residues D14, D58, T86 en K106 (numbered with respect to ORF123), which are part of the essential active site of CheY (26) in which D58 can be phosphorylated.

ORF140 (420 bp) is located upstream of *cinR* and codes for a protein similar to a hypothetical protein (AAG2039) of *Halobacterium* sp. NRC-1 (51% identity), and an unknown protein of *Bacillus subtilis* (CAB11811) (39% identity). Upstream of ORF123, a Met-tRNA gene (74 bp) (tRNAscan-S.E. v. 1.11) is found with the anticodon (CAT) located between nucleotides 4364 and 4366 of the 5.3-kb *Eco*RI fragment. This gene shows perfect (100%) DNA sequence identity to the Met-tRNA gene of *R. leguminosarum* bv. *viciae* (AF210630) and a *M. loti* sequence (AP002999) and is similar to a *Rhizobium* sp. NGR234 sequence (AE000079) (91% identity). Analysis of the intergenic region between ORF123 and the Met-tRNA gene indicates the presence of a putative terminator sequence downstream of the tRNA gene (nucleotides 5002–5068, $\Delta G = -22.9$).

NMR Analysis of the Isolated Compound Produced by R. etli CinI—An NMR analysis of the compound produced by *R. etli* CinI was conducted. As a control, 3OH-$C_{14:1}$-HSL was synthesized (data not shown) and analyzed. The NMR data of the synthetic compound are in agreement with previously recorded data (13).

The ^{1}H NMR spectrum of the *R. etli* compound contains all characteristic signals of a 3-hydroxyacyl-homoserine lactone. Evidence for the homoserine lactone moiety is constituted by the signal at 6.33–6.24 ppm (amide NH) and the characteristic butyrolactone signals at 4.52, 4.47, 4.27, 2.77, and 2.10 ppm. The line shapes and splitting patterns are in good agreement with those of synthetic acyl-homoserine lactones. Moreover, the line at 3.98 ppm is similar to the CH(OH) resonance in 3OH-$C_{14:1}$-HSL. However, the characteristic double bond signals between 5 and 6 ppm observed for 3OH-$C_{14:1}$-HSL, as well as the signals around 2.00 ppm of the protons on adjacent carbon atoms are absent. On the basis of its chromatographic properties (TLC, HPLC), the *R. etli* HSL is likely to possess a long chain fatty acid group. This allows to tentatively assign the spectrum of the *R. etli* autoinducer produced by CinI to a saturated long chain 3-hydroxy-acyl-homoserine lactone, which is clearly different from the structure of *R. leguminosarum* 3OH-$C_{14:1}$-HSL. In the subsequent part we will refer to the *R. etli* autoinducer as 3OH-(slc)-HSL.

Gene Regulation of cinI and cinR—To study the cell density-dependent expression of the *cin* locus, *cinR-gusA* (pFAJ4011) and *cinI-gusA* (pFAJ4014) fusions were constructed. The *cinR* gene in pFAJ4014 was inactivated by site-directed mutagenesis. To determine whether a promoter is present in the *cinR-cinI* intergenic region, a second *cinI-gusA* (pFAJ4015) fusion, containing a 632-bp upstream region of *cinI* (Fig. 1), was also constructed. As shown in Fig. 2*A*, *cinI* expression from pFAJ4014 under free-living conditions in a wild-type background increased with the cell density and reached a plateau (1500–2400 units) as the culture entered into the stationary phase. *cinI* expression from pFAJ4015 displayed a similar cell density-dependent pattern of expression (Fig. 2*B*). However, two differences between the two fusions can be noticed. Firstly,

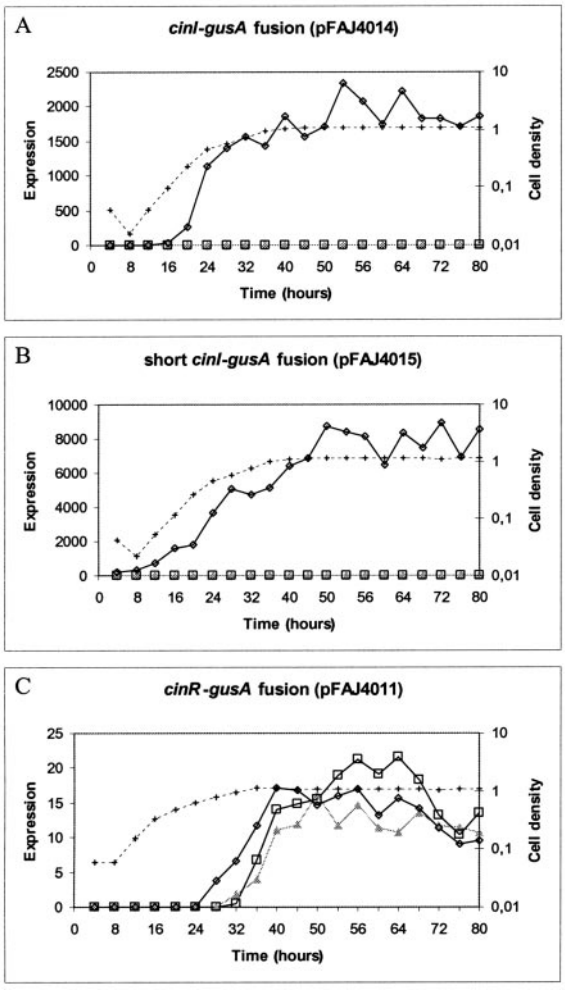

FIG. 2. **Expression of *cinR*- and *cinI-gusA* fusions.** *cinI* expression from pFAJ4014 (*A*) or pFAJ4015 (*B*) and *cinR* expression from pFAJ4011 (*C*) was examined quantitatively in a cell density-dependent manner. *Curves* representing the expression in the wild-type background are indicated by a *diamond*, in a *cinI* mutant background (FAJ4006) by a *gray triangle*, and in a *cinR* mutant background (FAJ4009) by a *square*. The absorbance at 595 nm of wild-type *R. etli* CNPAF512 containing pFAJ4011, pFAJ4014, or pFAJ4015 (*cross*) is shown. The growth of the other strains is similar to that of the wild type. The *graphs* are representative of experiments carried out independently several times.

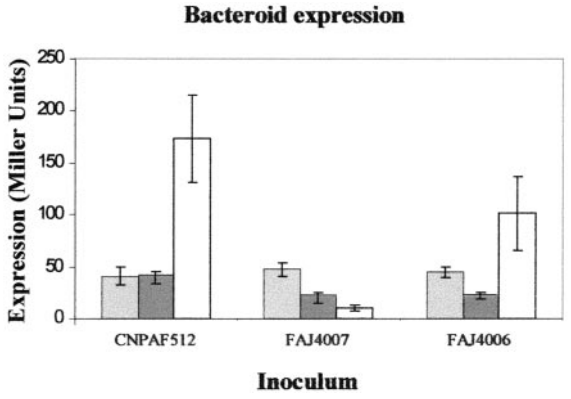

FIG. 3. **Expression of *cinR* and *cinI* during symbiosis.** Bacteroid expression of *cinR-gusA* (pFAJ4011, *light gray*) and *cinI-gusA* fusions (pFAJ4014, *dark gray*; pFAJ4015, *white*) was monitored in isolated bacteroids, obtained from 21-day-old bean nodules, induced by wild-type CNPAF512, the *cinR* (FAJ4007), and *cinI* (FAJ4006) mutants. Values are the means of at least nine plants. *Bars* represent mean ± S.D.

induction of *cinI* expression from pFAJ4015 starts at a lower absorbance compared with pFAJ4014. Secondly, the maximum expression level of *cinI* from pFAJ4015 is approximately 4-fold higher, compared with that of pFAJ4014. None of the *cinI-gusA* fusions are expressed in *cinR* or *cinI* mutant backgrounds (Fig. 2, *A* and *B*), demonstrating that transcription of *cinI* requires both CinI and CinR.

A threshold cell density (approximately $A_{595} = 0.6$) seems to be required to observe a very low *cinR* expression in wild-type and *cinR* or *cinI* mutant backgrounds (Fig. 2*C*). Although expression of *cinR* remains low, it reaches a maximum (~20 units) as soon as cells enter the stationary phase. The observation that both *cinR* and *cinI* expression is maximal during the same stage of growth is in agreement with a role of CinR in the regulation of *cinI* expression.

The observation that *cinI* is expressed in either the presence or absence of the *cinR* promoter region, demonstrates that *cinR* and *cinI* are likely organized into different transcriptional units. Furthermore, the overall high expression level of *cinI* (minimal 100-fold higher than *cinR*), suggests that transcription of *cinI* in both pFAJ4014 and pFAJ4015 is controlled by a promoter in the *cinR-cinI* intergenic region. This is in agreement with the presence of a putative terminator downstream of *cinR*. The difference in *cinI* expression levels between pFAJ4014 and pFAJ4015, is likely caused by a negative regulation at the level of the putative *cinI* promoter.

Expression under Symbiotic Conditions—Expression of the *cinI-gusA* and *cinR-gusA* fusions was monitored in isolated bacteroids, obtained from 21-day-old bean nodules, induced by wild-type CNPAF512, the *cinR* (FAJ4007), and *cinI* mutant (FAJ4006). The presence of the plasmid-borne *gusA*-fusions pFAJ4011, pFAJ4014, and pFAJ4015 in the different strains did not affect their symbiotic performance (data not shown). As illustrated in Fig. 3, expression of the *cinR* fusion (pFAJ4011) is low (~40 units) and does not significantly differ in the three genetic backgrounds. Expression of the short *cinI* fusion (pFAJ4015) is significantly higher (~8-fold) compared with the long *cinI* fusion (pFAJ4014) in wild-type background, similar to what was observed under free-living conditions. However, in contrast to free-living conditions, expression of the short *cinI* fusion is less dependent on the presence of CinI because *cinI* expression from pFAJ4015 is still observed in *cinI* mutant (FAJ4006) bacteroids. Similarly to free-living conditions, *cinI* gene expression requires the presence of CinR. Finally, it should be noted that the expression level of *cinI* in bacteroids is at least 10-fold lower compared with free-living conditions.

Shortly after inoculation (24 h), *cinI-gusA* expression (pFAJ4015) was observed on the surface of root hairs during colonization of wild-type and the *cinI* mutant (FAJ4006) (data not shown). Light microscopic analysis, 48 h after inoculation of the roots, localized pFAJ4015 expression in the infection tread, formed by wild-type and the FAJ4006 mutant (Fig. 4, *A* and *B*). However, no *cinI-gusA* expression from pFAJ4015 was observed in the *cinR* mutant (FAJ4007) during colonization (data not shown) or in the infection tread (Fig. 4*C*). Based on the intensity of the Gus-staining, the fusion seems less expressed in a *cinI* mutant background (FAJ4006) compared with the wild-type background, similarly as observed for bacteroid expression.

Analysis of Autoinducers—The cell-free culture supernatant of the *R. etli* wild type, the *cinR* and the *cinI* mutant, was extracted with ethyl acetate, and analyzed by TLC combined with the *A. tumefaciens tra* reporter system (3). As was previously published by Rosemeyer *et al.* (12), wild-type *R. etli* CNPAF512 produces at least seven autoinducers (Fig. 5, *lane A*). The *raiI* mutant, FAJ4010 (Fig. 5, *lane C*) produces 3OH-

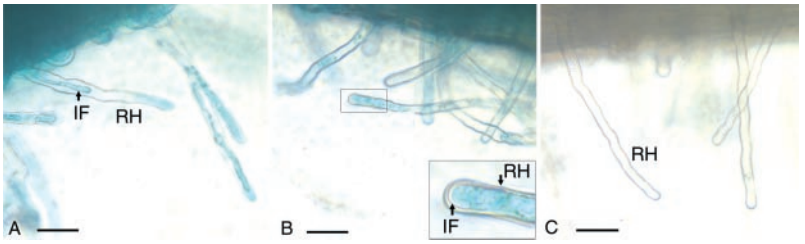

FIG. 4. **Light microscopic analysis of *cinI-gusA* (pFAJ4015) expression during the early steps of symbiosis.** *Phaseolus vulgaris* roots were inoculated with pFAJ4015/CNPAF512 (*A*), pFAJ4015/FAJ4006 (*cinI*) (*B*), pFAJ4015/FAJ4009 (*cinR*) (*C*). *RH*, root hair; *IF*, infection tread. The scale of the *bars* is 200 μM.

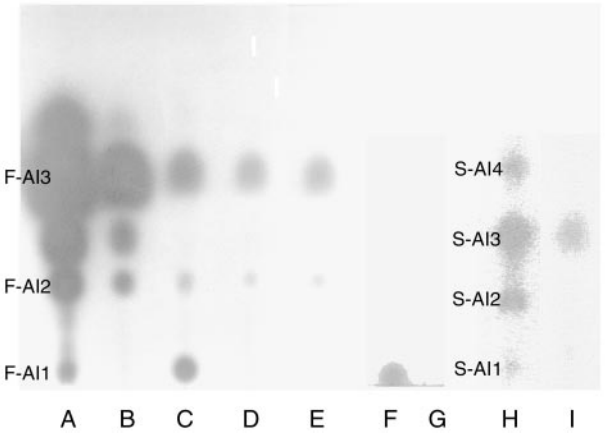

FIG. 5. **TLC analysis of *R. etli* autoinducers.** The ethyl acetate extracts were spotted on C_{18} RP-TLC plates and 60% MeOH was used as the liquid phase. Molecules with autoinducer activity were visualized with a soft agar overlay containing X-gal and the *A. tumefaciens* reporter strain. Autoinducers produced by free-living *R. etli* CNPAF512 (*A*), *cinI* mutant (FAJ4006) (*B*), *raiI* mutant (FAJ4010) (*C*), *raiIcinI* mutant (FAJ4013) (*D*), *A. tumefaciens* containing *R. etli* CNPAF512 *cinR* and *cinI* (pFAJ4013) (*F*) and the *A. tumefaciens* negative control (*G*). Autoinducer F-AI1 corresponds to 3OH-(slc)-HSL and F-AI2 and F-AI3 to spots also detected in non-inoculated TY medium (*lane E*). The autoinducers S-AI1 to S-AI4 produced by *R. etli* bacteroids and by root material are shown in *lanes H* and *I*, respectively.

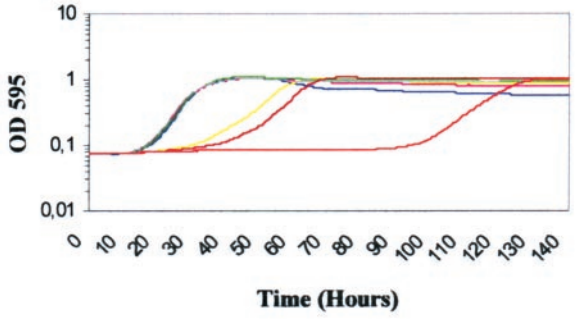

FIG. 6. **Growth of *R. etli* wild-type and autoinducer mutant strains.** Absorbance at 595 nm of *R. etli* (*blue*), the *raiI* mutant FAJ4010 (*pink*), the *raiR* mutant FAJ1329 (*yellow*), the non-polar *cinR* mutant FAJ4007 (*green*), the polar *cinR* mutant FAJ4009 (*gray*), the *cinI* mutant FAJ4006 (*red*), and the strongly delayed *raiIcinI* double mutant FAJ4013 (*orange*) were measured in a BioscreenC over a 6-day period.

(slc)-HSL (F-AI1) and two other active molecules (F-AI2, F-AI3), which co-migrate with compounds extracted from sterile, non-inoculated TY medium (Fig. 5, *lane E*). Ethyl acetate extracts from AMS medium or water were negative in this test (data not shown). Both *cinR* and *cinI* mutants (FAJ4006, FAJ4007, FAJ4009) secrete all of the autoinducers except 3OH-(slc)-HSL (Fig. 5, data shown for FAJ4006 in *lane B*) as was also observed in the growth inhibition assay. As illustrated in Fig. 5 *lane D*, the extract prepared from a *raiIcinI* double mutant FAJ4013, contains two active spots (F-AI2, F-AI3), co-migrating with active molecules present in sterile, non-inoculated TY. Taken together, these data suggest that two genetic systems, *rai* and *cin*, are required for the synthesis of all of the autoinducers by *R. etli* CNPAF512 as detected by the *Agrobacterium tra* reporter system.

To confirm that the *cin* locus is solely responsible for the production of the 3OH-(slc)-HSL molecule, a plasmid containing the 5.3-kb *Eco*RI fragment, pFAJ4013, was introduced in the *A. tumefaciens* NT1 strain (lacking the Ti plasmid, containing plasmid pJM749), a derivative lacking synthesis of autoinducers (Fig. 5, *lane G*). TLC analysis revealed the production of a compound co-migrating with the *R. etli* 3OH-(slc)-HSL (Fig. 5, *lane F*). Moreover, using this extract, growth inhibition of *R. leguminosarum* bv. *viciae* 248 was observed in a growth inhibition assay (data not shown).

Finally, the autoinducer production was analyzed during symbiosis. For this, the lysate, prepared from *R. etli* bacteroids, was extracted with ethyl acetate and the extracted autoinducers from one nodulated plant were analyzed on TLC for compounds activating the *A. tumefaciens tra* system. Less different autoinducers seem to be produced under symbiotic conditions compared with free-living growth (Fig. 5, *lane H*). One of the wild-type bacteroid autoinducer compounds, S-AI1, co-migrates on TLC with 3OH-(slc)-HSL (F-AI1). The bacteroid autoinducer S-AI3 co-migrates with a compound, extracted from root material and able to activate the *tra* reporter system (Fig. 5, *lane I*). Taken together, these results indicate that at least three different autoinducer molecules are produced by *R. etli* during symbiosis (S-AI1, S-AI2, S-AI4).

Mutation of the rai and cin Autoinducer System Affects Growth of Rhizobium—To examine the phenotypic relevance of the *rai* and *cin* system in *R. etli*, a *rai-cin* double mutant was constructed. To monitor growth, absorbance was measured in a BioscreenC over a 6-day period (total dilution 6000-fold). Growth of the *raiR* mutant (FAJ1329) and the *cinI* mutant (FAJ4006) was clearly delayed compared with wild-type growth in AMS mannitol medium as can be seen from Fig. 6. The lag phase was prolonged by 14.5 and 24.5 h, respectively. Noteworthy, the growth pattern of the *cinR* mutants, FAJ4007 and FAJ4009, as well as the growth of a *raiI* mutant (FAJ4010) was not different from that of the wild type (Fig. 6). Entry into the exponential phase was even more delayed (79 h more than the wild type) when growth of the *raiIcinI* double mutant, FAJ4013, was examined (Fig. 6). Furthermore, growth of the *cinI* ($g = 6.8$ h) and *raiR* ($g = 8.3$ h) mutants as well as that of the double *raiIcinI* mutant ($g = 8.8$ h) was marked by a 1.5- to 2-fold increase in generation time compared with the wild type ($g = 4.5$ h). The observed difference in lag phases was less pronounced when the culture was only 1000-fold diluted whereas no difference was obtained with a 100-fold-diluted preculture (data not shown). Although a similar increase in generation time was also observed when bacteria were grown in AMS succinate medium (data not shown), the difference in the lag phases was less pronounced, indicating that the *raicin*-dependent regulation of growth is complex and depends on the growth medium (carbon source) used.

Symbiotic Phenotype of cinR and cinI Mutants—The *R. etli* *cinR* and *cinI* mutants were tested for their ability to nodulate

TABLE I
Symbiotic phenotype

Each value is based on the examination of 7 plants inoculated with FAJ4013, 8 plants inoculated with FAJ4006 or FAJ4007, and 10 plants inoculated with CNPAF512 or FAJ4009. The standard deviation is indicated between parenthesis.

Strain	Kinetics of nodule appearance	Dry weight of plant	Dry weight of nodules	Number of nodules	Acetylene reduction
	day	*mg*	*mg*		*(μmol h^{-1})/plant*
CNPAF512	8.6 (2.2)	416.2 (136.6)	75.3 (25.0)	184.0 (34.5)	9.4 (2.0)
FAJ4006	8.3 (2.1)	310.3 (124.7)	86.6 (27.6)	248.0 (28.9)	2.8 (1.2)
FAJ4007	6.2 (1.1)	370.0 (95.7)	102.2 (25.2)	235.2 (54.9)	3.8 (1.0)
FAJ4009	7.9 (2.6)	345.9 (81.1)	102.4 (24.7)	236.1 (48.0)	2.9 (0.6)
FAJ4013	6.6 (1.1)	325.4 (55.4)	85.5 (19.9)	188.7 (48.8)	2.5 (1.1)

common bean (*Phaseolus vulgaris* cv. Limburgse vroege) and to fix nitrogen. No significant differences in kinetics of appearance of the first nodules were observed (Table I). Moreover, the plant and nodule dry weight as well as the nodule number, determined 21 days after inoculation, were not significantly different between plants nodulated by the wild-type or the mutant strains (Table I). However, a clear impact of the *cin* system on nitrogen fixation could be observed because inoculation of bean plants with the *R. etli cinR* mutants (FAJ4007 and FAJ4009) or the *cinI* mutant (FAJ4006) resulted in a statistically lower acetylene reduction activity (ARA) per plant (30–40% of wild-type ARA; Table I). Furthermore, inoculation with the FAJ4013 *raiIcinI* double mutant decreased nitrogen fixation per plant even further (27% of wild-type ARA; Table I). Autoinducer production, growth, and the tested symbiotic features are similar for FAJ4009 and FAJ4007, the polar and non-polar *cinR* mutant. This observation supports further the hypothesis that *cinR* and *cinI* are likely organized into different transcriptional units.

To further examine the effect of *cin* mutations at the bacteroid level, sections of nodules, formed by wild-type and mutant strains were analyzed by transmission electron microscopy (TEM). This analysis indicated that *cinI* mutant bacteroids were always individually packed in the symbiosome membrane (SM) (Fig. 7*A*), whereas wild-type symbiosomes usually contained multiple bacteroids (Fig. 7*B*). Furthermore, *cinI* mutant bacteroids were surrounded by a minimal symbiosome space (SS) compared with wild-type bacteroids. These results indicate that the *cin* system fulfills a key role during symbiosome development.

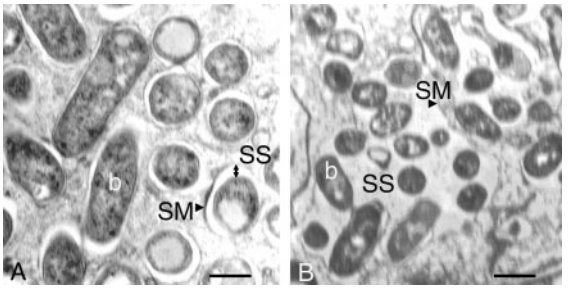

FIG. 7. **TEM of sections of 3-week-old nodules, formed by *R. etli* CNPAF512 (*B*) and the *cinI* mutant (FAJ4006) (*A*) analyzed in a Zeiss EM 900 electron microscope.** *SS*, symbiosome space; *SM*, symbiosome membrane; *b*, bacteroid. The scale of the *bars* is 0.6 μM.

DISCUSSION

We have characterized the *cin* locus in *R. etli* CNPAF512, involved in the synthesis of 3OH-(slc)-HSL, containing a saturated long acyl chain. In our current model, *cinI* codes for the autoinducer synthase and *cinR* for the transcriptional regulator that binds the 3OH-(slc)-HSL. The latter complex activates *cinI* expression. The chromatographic properties of 3OH-(slc)-HSL are very similar to 3OH-$C_{14:1}$-HSL, produced by *R. leguminosarum* bv. *viciae*. Moreover, both compounds induce growth inhibition of *R. leguminosarum* bv. *viciae* 248. In contrast, major differences between both *cin* loci with respect to growth under free-living conditions and symbiotic performance of the corresponding mutants were observed, indicating that both molecules may perform different functions in *R. etli* and *R. leguminosarum* bv. *viciae*.

Mutational and expression analysis revealed that *cinR* and *cinI* have distinct promoters. Expression of *cinR* is low both under free-living and symbiotic conditions and is cell density-dependent. Expression of *cinI* is also regulated in a cell density-dependent way and reaches a maximal expression level in the stationary phase. Furthermore, the *cinI* gene is expressed in bacteroids. Expression of *cinI* requires CinR both under free-living conditions and during symbiosis. Expression levels of *cinI* differ depending on the extent of the upstream region in the plasmid-borne *cinI-gusA* fusion construct. Possibly, the DNA sequence upstream of the putative *cinI* promoter in the plasmid construct competes for a trans-acting factor that is required for *cinI* transcription, such as CinR. Alternatively, several reports from the literature suggest complex regulation of genes involved in quorum-sensing, in particular the occurrence of negative regulators. Examples of such negative regulators are: EsaR in *Pantoea stewartii* (27), TraS in *A. tumefaciens* (28) and RsaL in *P. aeruginosa* (29). Moreover, Lithgow *et al.* (8) showed that expression of *cinI* is significantly reduced when the symbiotic plasmid pRL1JI is present in *R. leguminosarum* bv. *viciae*, resulting in a reduction in the level of 3OH-$C_{14:1}$-HSL. The mechanism of pRL1JI-mediated repression of *cinI* expression or the identity of the factors enhancing or relieving this repression have not yet been identified.

cinI is likely positively autoregulated in the bacteroids even though a clear expression level could be observed in a *cinI* mutant background. CinR may stimulate expression of *cinI* even in the absence of 3OH-(slc)-HSL. Whether CinR is activated through an autoinducer molecule produced by RaiI (see further) or whether a plant compound is able to activate CinR is yet unknown. Teplitski *et al.* (30) noticed that various species of higher plants can secrete substances, chemically different from bacterial AHLs but mimicking their activity. Also, extraction of non-inoculated *Phaseolus vulgaris* bean roots revealed the production of a compound activating the *Agrobacterium tra* reporter system, as illustrated in this work, making a cross-talk between both partners in the symbiosis quite reasonable. However, in contrast to the previously identified plant compounds (30), the *P. vulgaris* active molecules were found in the ethyl acetate fraction. Our suggestion of a possible cross-talk between a prokaryote and the eukaryotic host, was demonstrated in the case of *P. aeruginosa*, the opportunistic pathogen of immunocompromised individuals. The *P. aeruginosa* quorum-sensing signal molecule 3O-C_{12}-HSL stimulates interleukin-8 production in pulmonary epithelial cells (31) and may modulate the host immune response by suppressing cytokine production in macrophages (32). Up to now, several autoin-

ducer systems have been described in *Rhizobium* sp. but the *cin* system of *R. etli* is the first proven to be expressed in the infection tread and in differentiated bacteroids. In contrast, the *rhi* system, which is shown to be important for interaction with legumes and which is specific to *R. leguminosarum* bv. *viciae,* is only expressed in the rhizosphere (10). We have illustrated here for the first time that nitrogen-fixing bacteroids produce autoinducers in the nodules under conditions that are quite different from free-living growth. *In vivo* production and excretion of AHLs was also observed in lung tissues of mice infected with *P. aeruginosa* (33). Furthermore, autoinducers other than AHLs, such as quinolones (34) and diketopiperazines (DKP) (35), have been described in Gram-negative bacteria. The observation that DKP can be generated via non-enzymatic cyclization of linear dipeptides at extremes of pH and temperature (36) can offer an explanation for the presence of active compounds in sterile, non-inoculated TY medium.

Analysis of quorum-sensing in *R. etli* is further complicated by the fact that besides the *cin* system, involved in the synthesis of 3OH-(slc)-HSL, a second system, *rai*, regulates production of several other molecules with autoinducer activity (12). This is particularly striking when the effect of mutations in the *cin* or/and *rai* genes on growth of *R. etli* is analyzed: RaiR fulfills a key regulatory role, and it is shown that CinR negatively affects growth when 3OH-(slc)-HSL is not produced. Growth was even more delayed in a *raiIcinI* mutant. Both systems seem to interact at the level of unknown genes directly or indirectly controlling growth of the bacteria. The *R. etli* autoinduction systems clearly affect the symbiotic properties of the bacterium. The *rai* system is involved in restriction of the nodule number, whereas nitrogen fixation activity is not affected (12). On the other hand, the decreased acetylene reduction activity of plants nodulated by *cin* mutant strains and the bacteroid morphology, illustrate the important role the *cin* quorum-sensing system plays in *R. etli* during symbiosis. A disrupted communication, as observed in the *cin* mutants, results in an arrest of bacteroid differentiation. Also, in contrast to wild-type bacteroids, *cinI* mutants are individually enclosed in a symbiosome and are devoid of a large symbiosome space. The *cin* quorum-sensing system is a prerequisite to complete the differentiation cycle of the bacterium. In contrast, it was shown that 3OH-$C_{14:1}$-HSL, produced by CinI in *R. leguminosarum* bv. *viciae*, is not required for the formation of effective nodules (11). This observation was also confirmed by Lithgow *et al.*(8) in a plant experiment where mutation of *cinI* had little effect on growth or nodulation. In summary, *R. etli* 3OH-(slc)-HSL and *R. leguminosarum* bv. *viciae* 3OH-$C_{14:1}$-HSL share some common characteristics but elicit different phenotypic changes in their respective producing cells.

Acknowledgments—We thank Stephen Farrand and Anton van Brussel for providing the *A. tumefaciens* autoinducer system and *R. leguminosarum* bv. *viciae* strains, respectively; Johan Billen for assistance and access to the T.E.M. facility; Chuanwu Xi for technical assistance during the preparation of the microscopic samples; and Serge Buellens for helping with the plant experiments.

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