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

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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-hydroxyacyl-homoserine lactone, abbreviated as 3OH-(slc)-HSL. Using **cin-gus**A 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) 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<sub>6</sub>-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-(3R)-hydroxy-7-cis-tetradecenoyl-i-homoserine lactone (3OH-C<sub>14</sub>-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<sub>6</sub>-HSL and C<sub>8</sub>-HSL and decreased **rhiR** expression cause a repression of the rhizosphere-expressed genes in **cinI** or **cinI** mutants (8–10). Furthermore, 3OH-C<sub>14</sub>-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

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weight bacteriocin “small”, which is common in fast-growing rhizobia. The properties, growth inhibition, and autoinducer activity, are features reported for 3OH-C14,1-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-(scl)-HSL (scl, 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

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 EcoRI deletions procedures (Erase-A-Base) (15). 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 EcoRI fragment from pFAJ4000 containing cinR, cinI, orf123, and orf140 was first cloned into pBluescriptIKs’ yielding pFAJ401. A PstI-EcoRI fragment of pFAJ4003, lacking the 5’-end of orf140, was EcoRI-PstI subcloned in the broad host range vector pLAFR3 (pFAJ4012; Fig. 1). Furthermore, pFAJ4013 was made by insertion of the 5.3-kb EcoRI fragment from pFAJ4003 in pPZ2P200, in which part of the multiple cloning site between XhoI and PacI was deleted.

A fragment containing cinR and cinI was amplified via PCR using primers Rhi15 (5’-AGTGGAATTCATCCATGGCAGAGGATAC-3’) and Rhi16 (5’-TAGGAGATTCTCGTACATCATCATACCTCGC-3’) and pFAJ4003 as template DNA. The resulting 3.3-kb fragment was digested with EcoRI and BamHI and cloned in pUCN01 derivative lacking the SplI recognition site via restriction digest and blunt ligation yielding plasmid pFAJ4004 (Fig. 1). The 2.2-kb BamHI fragment from pHP450-Km containing CinR gusA cassette was ligated into the unique SplI site of cinI in pFAJ4004 before blotting of the fragments.

The cinR::cinI-Km locus was further cloned into the sacB suicide vector pJQ200uc1 as a 5.2-kb NotI fragment to obtain pFAJ4006. The non-polar cinR point mutation and an additional frameshift were introduced via the QuickChange™ site-directed mutagenesis (Stratagene) using primers Rhi52 (5’-CATGCGCATCTGACACGAGAACG-3’) and Rhi33 (5’-CGTCTCTGATGATTGGATGGG-3’) and Rhi33 (5’-CGTCTCTGATGATTGGATGGG-3’) on pFAJ4004. As a result of the mutation, a new and unique KpnI site (GTTCCT; mutations are shown in bold: insertion of A; substitution G with C) was created in cinR. Subsequently, the 3.3-kb NotI fragment containing the mutated cinR gene was inserted into pJQ200uc1 creating pFAJ4007. The cinR::Sp fusion mutation was introduced by introducing the recombinant bacteriocin resistance cartridge from pHP450 into the unique KpnI site within cinR of pFAJ4007 after blotting 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 pWM-TraI was inserted into the cinR locus of pFAJ4007 via blunt-end ligation resulting in pFAJ4008. For the construction of a R. etli traI 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 traI::cinI::Km double mutant FAJ4013.

RESULTS

Identification of the cin Locus of R. etli CNPAF512—a Tn5-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. The cinR promoter region was amplified by PCR with FAJ1337 as a template using primers Rhi15 and Tn5-B (5’-GGTCCGTGGTACGAGCACGCAT-3’) and FAJ1337 with primers Rhi15 and Tn5-B (5’-GGTCCGTGGTACGAGCACGCAT-3’) yielding Plasmid pFAJ4014 (cin-gus fusion carrying a mutant cinR gene region), the 1.8-kb EcoRI-SphI fragment of pFAJ4007 was blunt-end ligated into the HindIII site of pFAJ1327 containing (7) (7) after blunting of the vector, yielding plasmid pFAJ4010. For the construction of a R. etli traI 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 traI::cinI::Km double mutant FAJ4013.

Concentration of Fusion—Plasmid-borne PcinR:GusA and PcinI:GusA fusions were constructed in the promoter probe vector pFAJ1703 (20). The promoter region was amplified by PCR with FAJ1337 as a template using primers Rhi15 and Tn5-B (5’-GGTCCGTGGTACGAGCACGCAT-3’) and FAJ1337 with primers Rhi15 and Tn5-B (5’-GGTCCGTGGTACGAGCACGCAT-3’) yielding Plasmid pFAJ4014 (cin-gus fusion carrying a mutant cinR gene region), the 1.8-kb EcoRI-SphI fragment of pFAJ4007 was blunt-end ligated into the HindIII site of pFAJ1327 containing (7) (7) after blunting of the vector, yielding plasmid pFAJ4010. For the construction of a R. etli traI 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 traI::cinI::Km double mutant FAJ4013.

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DNA sequence analysis of the cloned 5.3-kb R. leguminosarum complemented for growth inhibition of the sensitive strain pLAFR1 (21). A 5.3-kb EcoRI 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 EcoRI 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 preferred codon usage (searchcatt, 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 (AAFF89989), 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, ΔG = −26.3). PCR analysis with a Tn5-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 (AAFF89990), it is also similar to CerI (33% identity) of R. sphaeroides (AAC46022) and RaiI of R. etli (39% identity) (AAC38173). The putative protein with a calculated molecular mass of 55.0 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, ΔG = −23.8; nucleotide 3694–3735, ΔG = −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 (AAFF89991), 40% identity to a probable response regulator of Mesorhizobium loti (BABB49462) and 35% identity to the FixL receiver domain of R. etli (AAOG00949). 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 (AAOG2039) 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) (tRNAscane-S.E. v. 1.11) is found with the anticodon (CAT) located between nucleotides 4364 and 4366 of the 5.3-kb EcoRI 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, ΔG = −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-C14:1-HSL was synthesized (data not shown) and analyzed. The NMR data of the synthetic compound are in agreement with previously recorded data (13).

The 1H 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-C14:1-HSL. However, the characteristic double bond signals between 5 and 6 ppm observed for 3OH-C14:1-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-C14:1-HSL. In the subsequent part we will refer to the R. etli autoinducer as 3OH-(iso)-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. 2A, 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. 2B). However, two differences between the two fusions can be noticed. Firstly,
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. 2C). 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 (FAJ4006) mutants. Values are the means of at least nine plants. Bars represent mean ± S.D.
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 \( rai \) mutant (FAJ4329) and the \( cin \) 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 \( cin \) mutants, FAJ4007 and FAJ4009, as well as that of the \( rai \) 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 \( rai\)-\( cin \) double mutant, FAJ4013, was examined (Fig. 6). Furthermore, growth of the \( cin \) (\( g = 6.8 \) h) and \( rai \) (\( g = 8.3 \) h) mutants as well as that of the double \( rai\)-\( cin \) 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 \( rai \)-dependent regulation of growth is complex and depends on the growth medium (carbon source) used.

Symbiotic Phenotype of \( cin \) Mutants—The \( R. \) \( etli \) \( cin \) and \( cin \) mutants were tested for their ability to nodulate...
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common bean (Phaseolus vulgaris cv. Limburgerse 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. 7A), whereas wild-type symbiosomes usually contained multiple bacteroids (Fig. 7B). 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.

**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-C12-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-C14:1-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. Tepelitk 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 30-C12-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 thread 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-(srl)-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 *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-(srl)-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 acetyl 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}\text{-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-(srl)-HSL and *R. leguminosarum* bv. *viciae* 3OH-C_{14:1}\text{-HSL} share some common characteristics but elicit different phenotypic changes in their respective producing cells.

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