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raiIR Genes Are Part of a Quorum-Sensing Network Controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*

F. Wisniewski-Dyé,[1]† J. Jones,[1] S. R. Chhabra,[2] and J. A. Downie[1]*

John Innes Centre, Norwich NR4 7UH,[1] and School of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD,[2] United Kingdom

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Analysis of *N*-acyl-L-homoserine lactones (AHLs) produced by *Rhizobium leguminosarum* bv. viciae indicated that there may be a network of quorum-sensing regulatory systems producing multiple AHLs in this species. Using a strain lacking a symbiosis plasmid, which carries some of the quorum-sensing genes, we isolated mutations in two genes (*raiI* and *raiR*) that are required for production of AHLs. The *raiIR* genes are located adjacent to *dad* genes (involved in D-alanine catabolism) on a large indigenous plasmid. RaiR is predicted to be a typical LuxR-type quorum-sensing regulator and is required for *raiI* expression. The *raiR* gene was expressed at a low level, possibly from a constitutive promoter, and its expression was increased under the influence of the upstream *raiI* promoter. Using gene fusions and analysis of AHLs produced, we showed that expression of *raiI* is strongly reduced in strains carrying mutations in *cinI* or *cinR*, genes which determine a higher-level quorum-sensing system that is required for normal expression of *raiIR*. The product of CinI, *N*-(3-hydroxy-7-*cis* tetradecenoyl) homoserine lactone, can induce *raiR*-dependent *raiI* expression, although higher levels of expression are induced by other AHLs. Expression of *raiI* in a strain of *Agrobacterium* that makes no AHLs resulted in the identification of *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3OH,C_8-HSL) as the major product of RaiI, although other AHLs that comigrate with *N*-hexanoyl-, *N*-heptanoyl-, and *N*-octanoyl-homoserine lactones were also made at low levels. The *raiI* gene was strongly induced by 3OH,C_8-HSL (the product of RaiI) but could also be induced by other AHLs, suggesting that the *raiI* promoter can be activated by other quorum-sensing systems within a network of regulation which also involves AHLs determined by genes on the symbiotic plasmid. Thus, the *raiIR* and *cinIR* genes are part of a complex regulatory network that influences AHL biosynthesis in *R. leguminosarum*.

Many plant-associated bacteria regulate gene expression in a cell density-dependent manner by using quorum sensing via *N*-acyl homoserine lactones (AHLs) (7). These AHLs pass out of, and into, bacterial cells. As the population of bacteria increases, so does the concentration of AHLs (38). Once the AHLs reach a threshold concentration, they act as coinducers to induce gene expression. Three types of AHL synthases, corresponding to LuxI, LuxM and HtdS types, have been identified (see reference 32). Often, the enzyme that is involved in AHL production can be induced by the AHL it produces, thereby creating a positive feedback loop that induces yet higher levels of AHLs. Genes that are regulated via such quorum-sensing-based systems in plant-associated bacteria include genes involved in plasmid transfer, antibiotic production, secretion of enzymes involved in pathogenesis, and production of various secondary metabolites, such as pyocyanin and pyoverdin (38).

Species of legume-nodulating rhizobia are included in this diverse group of bacteria that use AHL-based quorum-sensing systems. In a survey of AHLs produced by diverse soil bacteria, it was noted that some *Rhizobium* spp. produced the greatest diversity of quorum-sensing signaling molecules that were detected following thin-layer chromatography (TLC) (7). In *Rhizobium leguminosarum* bv. viciae many different AHLs are produced, and it was predicted that this diverse range of AHLs could be due to at least four separate loci involved in AHL production (17). In the *Sinorhizobium meliloti* database (http ://sequence.toulouse.inra.fr/meliloti.html), one AHL synthase gene (encoding a LuxI-like protein) and multiple *luxR*-like genes, two of which regulate motility genes, are predicted (30). In the *Mesorhizobium loti* database (http://www.kazusa.or.jp /rhizobase/index.html), there are three predicted LuxI-like proteins and multiple LuxR-like regulators, but their roles are not known. In *Rhizobium etli* at least two separate AHL production genes have been predicted to be present, one of which, *raiI*, has been shown to determine the production of several undefined AHLs (25). In *R. leguminosarum* bv. viciae, two AHL production loci are thought to be located on the symbiotic plasmid pRL1JI. One of these (*traI*) produces multiple undefined AHLs and is involved with plasmid transfer (17, 37), while the other (*rhiI*) is involved in the production of AHLs that induce the rhizosphere-expressed genes *rhiABC,* in association with the regulator RhiR (24). The *rhiABC* genes play an undefined role in nodulation; in some genetic backgrounds, mutation of the *rhi* genes can decrease nodulation (9). Expression of *rhiI* is *rhiR* dependent and is positively autoregulated by the AHLs *N*-hexanoyl-L-homoserine lactone (C_6-HSL), *N*-heptanoyl-L-homoserine lactone (C_7-HSL), and *N*-octanoyl-L-homoserine lactone (C_8-HSL), all of which are produced by RhiI (24).

Besides *rhiI* and *traI*, there is evidence for two other AHL synthases, neither of which is on the symbiosis plasmid (17).

* Corresponding author. Mailing address: John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom. Phone: 1603 450207. Fax: 1603 450045. E-mail: allan.downie@bbsrc.ac.uk.

† Present address: Laboratoire d'Ecologie Microbienne, UMR CNRS 5557, Université Claude Bernard Lyon I, Villeurbanne, France.

One of these is encoded by *cinI*, which is on the chromosome, and is regulated by the product of the adjacent gene, *cinR* (17). Mutations in *cinI* or *cinR* reduce, but do not block, *rhiI* expression, and there is a net decrease in levels of RhiI-made AHLs (17). Therefore, the *cinRI* locus imposes a higher level of control of *rhiI* and *rhiABC* expression. CinI produces *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (3OH,$C_{14:1}$-HSL), which does not directly induce *rhiI*; the effect of this AHL on *rhiI* gene expression appears to be indirect.

In addition to decreasing the levels of RhiI-made AHLs, mutation of *cinR* or *cinI* decreased the levels of various other AHLs made by *R. leguminosarum* bv. viciae. In a strain lacking a symbiotic plasmid (and hence *rhiI* and *traI*), multiple AHLs are made in addition to 3OH,$C_{14:1}$-HSL, the only detected product of CinI (17, 24). The fact that mutation of *cinI* in a strain lacking a symbiotic plasmid abolished the production of 3OH,$C_{14:1}$-HSL but only reduced the production of the other AHLs (17) implies that there is another locus involved in the production of these AHLs.

AHLs in *R. leguminosarum* strains have been proposed to be involved in stationary-phase adaptation and maintenance of viability in stationary-phase cultures. Thus, the product of CinI (3OH,$C_{14:1}$-HSL) inhibited the growth of some strains of *R. leguminosarum* bv. viciae and in fact was previously known as "small bacteriocin" because of its growth-inhibiting properties (13). Gray et al. (12) showed that this was due to growth arrest and proposed that the growth inhibition may be due to a conversion of exponential-phase cells to nongrowing stationary cells. Subsequently, added 3OH,$C_{14:1}$-HSL was shown to confer long-term viability on cultures of *R. leguminosarum* that had not adapted to stationary phase (33).

In this work we have characterized a nonsymbiotic-plasmid-borne locus (*raiIR*) involved in production of AHLs, and we show that it is part of a quorum-sensing network.

MATERIALS AND METHODS

Microbiological techniques. *Rhizobium* and *Agrobacterium* strains were grown at 28°C in TY medium (4), and *Escherichia coli* was grown at 37°C in L medium (26). Antibiotics were added as appropriate to maintain selection for plasmids. Bacterial growth was monitored at 600 nm using an MSE Spectroplus spectrophotometer. β-Galactosidase activities were measured (21) using a Titertek Multiscan Plus spectrophotometer. When added, AHLs were added at the start of growth to a final concentration of 20 nM or 1 μM. The AHLs C_6-HSL, C_7-HSL, C_8-HSL, *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3OH,C_8-HSL), 3OH,$C_{14:1}$-HSL, *N*-(3-oxooctanoyl)-L-homoserine lactone (3O,C_8-HSL), and *N*-(3-oxohexanoyl)-L-homoserine lactone (3O,C_6-HSL) were synthesized essentially as described previously (8).

Nodulation tests were done using peas (*Pisum sativum* L.) of the Wisconsin Perfection variety as described previously (5), using a minimum of 16 matched plants per test; at least two separate tests were carried out with similar results.

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *R. leguminosarum* strain 8401 lacks a symbiotic plasmid, and all *Rhizobium* strains used are derived from 8401. A34 is a derivative of 8401 carrying the symbiotic plasmid pRL1JI. Plasmids were mobilized into *Rhizobium* and *Agrobacterium* spp. by triparental matings using a helper plasmid. For genetic complementation studies, a cosmid library of *R. leguminosarum* A34 DNA cloned in pLAFR1 (15) was transferred into mutants by filter mating and selection of tetracycline-resistant colonies.

Strain 8401 was mutagenized with Tn*5-gus* by using *E. coli* strain MM294/pRK600::Tn*5-gusA1* as a donor of the suicide plasmid pRK600::Tn*5-gusA1* essentially as described previously (27). A population of about 8,000 colonies was screened for impaired AHL production by picking colonies onto a lawn of the AHL biosensor strain *Chromobacterium violaceum* CV026 (20) to identify mutants that did not induce the purple pigment violacein. Strain A700 was one such mutant, and small-bacteriocin tests revealed that A700 retained the ability to produce small bacteriocin (3-OH,$C_{14:1}$-HSL). Unfortunately, transduction tests revealed that the Tn*5* in A700 was not linked to the defect in AHL production, so the mutant phenotype was complemented by using a cosmid library, leading to the identification of pIJ9001 and subsequently pIJ9228, containing the subcloned *raiIR* region that complemented the mutation.

Mutagenesis of plasmids pIJ9001 and pIJ9228 was carried out using phage λ carrying Tn*5* (29). The Tn*5*-containing derivatives of pIJ9001 were mated into a rifampin-resistant derivative of 8401, and the transconjugants were screened for low levels of AHL production. Four mutant cosmids, pIJ9161, pIJ9163, pIJ9164, and pIJ9165, were isolated; from each of these, part of Tn*5* and the flanking DNA were subcloned with *Bam*HI into pBluescript, and the precise sites of Tn*5* insertions were identified by DNA sequencing using a Tn*5*-specific primer. The mutated derivatives of pIJ9228 were mated into a rifampin-resistant derivative of *Agrobacterium* strain C58.00, and the transconjugants were screened for inability to activate pigment production by *C. violaceum* CV026. Two of the resulting plasmids, pIJ9231 and pIJ9234, carrying Tn*5* in *raiI* and *raiR,* respectively, were selected, and the sites of Tn*5* insertion were identified by DNA sequencing.

Tn*5*-containing fragments from pIJ9231 and pIJ9234 were subcloned with *Apa*I-*Xba*I into the *sacB* suicide vector pJQ200KS (23). The *sacB* gene confers lethal susceptibility to sucrose, allowing for selection of recombinants. The *raiI7*::Tn*5* and *raiR8*::Tn*5* mutations were recombined from the plasmids into strain 8401 to form A789 and A802, obtained by selecting for kanamycin-resistant, sucrose-resistant colonies (23). The mutations in A789 (*raiI7*::Tn*5*) and A802 (*raiR8*::Tn*5*) were then transduced into A34 by using phage RL38 (6) to produce A793 and A803, respectively. To generate a derivative of 8401 defective in both *cinI* and *raiI*, the *raiI7*::Tn*5* mutation was transduced into the *cinI* mutant A643 to generate A797. To create a double mutant in a strain carrying pRL1JI, the *raiI7*::Tn5 mutation was transduced into A664 to generate A798, which is a derivative of A34 carrying mutations in both *cinI* and *raiI*.

The *raiIR* gene region was subcloned from pIJ9001 into pBBR1MCS-5 (14) on an 8.5-kb *Eco*RI fragment to make pIJ9222. A 2.3-kb *Bam*HI fragment carrying *raiIR* was subcloned in pBBR1MCS-5 to form pIJ9228. Plasmid pIJ9276, containing only *raiR*, was obtained by deleting a *Sac*I fragment from pIJ9228 by using a *Sac*I site at the end of *raiI* and a *Sac*I site in the vector.

To construct pIJ9280 (*raiI-lacZ*), a 1.35-kb *Eco*RI-*Mfe*I fragment from pIJ9228 was cloned into pMP220 (31) cut with *Eco*RI. To make pIJ9271 and pIJ9272 (*raiIR-lacZ* and *raiR-lacZ*), a 1.8-kb *Eco*RI-*Cla*I fragment was first subcloned from pIJ9228 into pBluescript and then subcloned as a 1.8-kb *Eco*RI-*Kpn*I fragment (still carrying *raiI*) or a 0.5-kb *Mfe*I-*Kpn*I fragment (not carrying *raiI*) into pMP220 cut with *Eco*RI and *Kpn*I.

Molecular biology techniques. DNA cloning, ligation, transformation, restriction enzyme mapping, and DNA hybridization were performed by standard methods (26). Sequencing reactions were carried out by using the Amersham "Big Dye" kits and an Applied Biosystems automated sequencer (ABI 377). DNA sequencing of the *raiIR* genes was carried out on both strands by using primer walking on pIJ9225, which is a derivative of pBluescript carrying the 2.3-kb *Bam*HI fragment. Other DNA sequences were determined by using pIJ9220 (which carries the 8.5-kb *Eco*RI fragment in pBluescript) or various derivatives of pIJ9220 carrying subcloned fragments. Database searches of the predicted protein sequences were carried out by using the BLAST and FASTA (2) programs to find related sequences in the EMBL and SwissProt protein sequence databases.

Assay of AHLs. *Rhizobium* and *Agrobacterium* cultures were grown for 62 and 48 h, respectively, in TY medium to optical density at 600 nm (OD_{600}) readings of approximately 0.9. Cells were removed by centrifugation, and AHLs were extracted from culture supernatants, as described previously (39). AHLs were analyzed by TLC using *Agrobacterium tumefaciens* NT1/pZLR4 (7, 28) or *C. violaceum* CV026 as the AHL indicator organism (20). Extracts of culture supernatants were spotted onto aluminum-backed RP18 reverse-phase TLC plates (Merck) and dried in a stream of air. Samples were separated by using 60% (vol/vol) methanol in water as the mobile phase. Once the solvent front had migrated to within 2 cm of the top of the chromatogram, the plate was removed from the chromatography tank and dried in air. Because *Rhizobium* cultures produce several closely related AHLs, the separation process was repeated so as to achieve adequate separation of AHLs. After being dried for the second time, the chromatogram was overlaid with a thin film of either soft L agar (0.7% [wt/vol]) seeded with *C. violaceum* CV026 or soft AB agar (28) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and seeded with *A. tumefaciens* NT1/pZLR4.

For electrospray mass spectrometry (ES-MS) of AHLs, *Agrobacterium* strain A58.00 carrying pIJ9228 (*raiIR*) was grown in 2 liters of AB medium (28) and the cells were pelleted by centrifugation. The spent culture supernatant

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
R. leguminosarum		
8401	Strain lacking a symbiotic plasmid; Str^r	15
A34	Like 8401 but containing pRL1JI (pSym)	10
A643	8401 carrying *cinI3*::Spc	This work
A644	A34 carrying *cinI3*::Spc	This work
A700	Mutant of 8401; probably *raiI*::IS*50*	This work
A789	8401 carrying *raiI7*::Tn*5*	This work
A793	A34 carrying *raiI7*::Tn*5*	This work
A797	8401 carrying *raiI7*::Tn*5* and *cinI3*::Spc	This work
A798	A34 carrying *raiI7*::Tn*5* and *cinI3*::Spc	This work
A802	8401 carrying *raiR8*::Tn*5*	This work
A803	A34 carrying *raiR8*::Tn*5*	This work
Agrobacterium sp.		
C58.00	Lacks AT and Ti plasmids; AHL negative	35
NT1/pZLR4	*traG-lacZ*-based AHL detection strain	7
C. violaceum CV026	AHL detection strain	20
Plasmids		
pIJ9001	Cosmid carrying *raiIR* region	This work
pIJ9161	pIJ9001 carrying *raiR5*::Tn*5*	This work
pIJ9163	pIJ9001 carrying *raiR3*::Tn*5*	This work
pIJ9164	pIJ9001 carrying *raiR4*::Tn*5*	This work
pIJ9165	pIJ9001 carrying *raiR6*::Tn*5*	This work
pIJ9220	*raiIR* on 8.5-kb *Eco*RI fragment from pIJ9001 in Bluescript(KS)	This work
pIJ9222	8.5-kb fragment from pIJ9222 in pBBR1MCS-5	This work
pIJ9225	*raiIR* on 2.3-kb *Bam*HI fragment in Bluescript(KS)	This work
pIJ9228	*raiIR* on 2.3-kb *Bam*HI fragment in pBBR1MCS-5	This work
pIJ9231	pIJ9228 carrying *raiI7*::Tn*5*	This work
pIJ9234	pIJ9228 carrying *raiR8*::Tn*5*	This work
pIJ9271	*raiIR-lacZ* in pMP220	This work
pIJ9272	*raiR-lacZ* in pMP220	This work
pIJ9276	*raiR* cloned in pBBR1MCS-5	This work
pIJ9280	*raiI-lacZ* in pMP220	This work
pBBR1MCS-5	Broad-host-range cloning vector	14
pMP220	Broad-host-range *lacZ* expression vector	31

was pumped through a C_{18} reverse-phase column (Sep Pak; Waters Corporation). The column was washed with 20 ml of H_2O, and the AHLs were eluted with 3 ml of acetonitrile, which was then evaporated off under a stream of N_2 gas. The sample was redissolved in 100 μl of acetonitrile and separated by TLC as described above. Part of the chromatograph was developed, and bands corresponding to the detected components were scraped off the remaining part of the TLC. The silica was extracted twice with 3 ml of acetone, which was then evaporated off under N_2. Samples were redissolved in 100 μl of a 1:1 mixture of acetonitrile and water to which was added formic acid to a final concentration of 0.5% (vol/vol). The sample (20 μl) was introduced into the electrospray source (Platform VG Instruments) at a flow rate of 10 μl min^{-1}. Spectra were recorded in the positive mode at a speed of 10 s for *m/z* 100 to 400 with a core voltage of 40 V. Spectra were processed by using Masslynx, version 2.0, software and compared with chemically synthesized standards for C_6-HSL, 3O,C_8-HSL, and 3OH,C_8-HSL.

Plasmid hybridization. *Rhizobium* cells were cultured, prepared for in-gel lysis, and loaded in a horizontal back-trap agarose gel (0.8%) to separate high-molecular-weight plasmids (36). After 16 h at 100 V, the gel was transferred to a Hybond N membrane (Amersham). To probe the *raiIR* region, a 1.35-kb gel-purified *Eco*RI-*Mfe*I fragment carrying part of *raiI* was labeled with [α-^{32}P]dCTP by using the Rediprime II kit (Amersham). The membrane was hybridized overnight at 60°C and then washed twice for 15 min each time with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) and twice for 10 min each time with 2× SSC–0.2% SDS.

Nucleotide sequence accession number. The sequence of the *raiIR* gene region has been deposited in the EMBL database under accession no. AJ427969.

RESULTS AND DISCUSSION

Identification of the *raiIR* genes involved in AHL production. *R. leguminosarum* strain 8401, which lacks a symbiosis plasmid, produces several AHLs (Fig. 1 and 2). The *C. violaceum* CV026 detection system reveals three strongly reacting spots (Fig. 1, lane a), whereas the *Agrobacterium traG-lacZ* detection system reveals at least five components (Fig. 2, lane d). *C. violaceum* CV026 does not detect 3OH,$C_{14:1}$-HSL (the product of CinI) (Fig. 1), and this enabled us to screen for mutants defective in the production of AHLs determined by genes other than *cinI*. One mutant, A700, lacked CV026-detectable AHLs (Fig. 1, lane b) but produced normal levels of 3OH,$C_{14:1}$-HSL based on a bacteriocin type bioassay (data not shown), and therefore the mutation did not affect the expression of *cinI* or *cinR*.

Plasmids that restored AHL production were isolated by conjugating cosmids into A700 and screening for transconjugants that restored activation of pigment production by *C. violaceum* CV026. Two classes of complementing clones were isolated, one corresponding to the previously identified *rhiI* gene region from pRL1JI and one novel plasmid that was called pIJ9001. As shown in Fig. 1, lane c, pIJ9001 restored

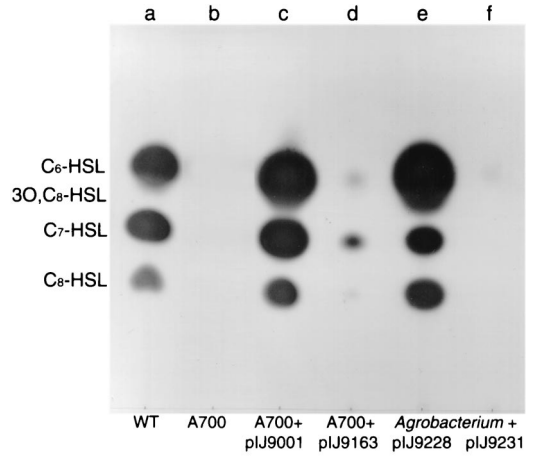

FIG. 1. Identification of AHLs produced by *raiI*. *Rhizobium* and *Agrobacterium* cultures were grown in 100 ml of TY broth for 62 and 48 h, respectively. AHLs extracted from cell-free culture supernatants were separated by TLC and detected by using an overlay of agar seeded with *C. violaceum* CV026. Each lane was loaded with a volume of extract equivalent to 25 ml of culture. Lanes a and b, extracts from *R. leguminosarum* strain 8401 and mutant A700, respectively; lanes c and d, extracts from mutant A700 complemented with cosmids pIJ9001 and pIJ9163 (*raiR3*::Tn*5*), respectively; lanes e and f, extracts from *A. tumefaciens* C58.00 containing pIJ9228 and pIJ9231 (*raiI7*::Tn*5*), respectively. The migration positions of standards (not shown) are indicated to the left of the chromatogram.

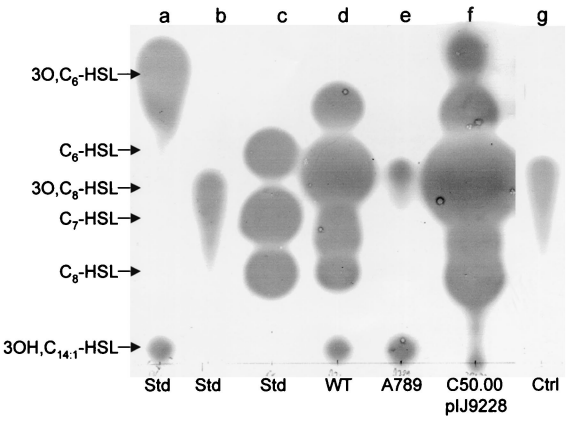

FIG. 2. Identification of AHLs produced by RaiI. Cultures were grown in 100 ml of TY broth. AHLs extracted from cell-free culture supernatants were separated by TLC and detected by using an overlay of agar seeded with *A. tumefaciens* carrying *traG-lacZ* (NT1/pZLR4) as a detection system. Lane a, standards 3O,C_6-HSL and 3OH,$C_{14:1}$-HSL; lane b, standard 3O,C_8-HSL; lane c, standards C_6-HSL, C_7-HSL, and C_8-HSL; lanes d and e, extracts (equivalent to 5 ml of culture) from *R. leguminosarum* 8401 and A789 (*raiI7*::Tn*5*), respectively; lane f, extract (equivalent to 2.5 ml of culture) from *A. tumefaciens* C58.00 containing pIJ9228 (*raiIR*); lane g, extract (equivalent to 5 ml of culture) of sterile growth medium in which bacteria had not been grown, used as a control.

A700 to a wild-type pattern of AHL production, based on activation of purple pigment in CV026. DNA hybridization experiments confirmed that pIJ9001 did not contain DNA from the symbiosis plasmid pRL1JI (data not shown).

Plasmid pIJ9001 was mutagenized with Tn*5*, and several mutant derivatives (e.g., pIJ9163) in which AHL production was affected were identified (Fig. 1, lane d). The Tn*5* insertions were located by mapping and DNA sequencing at different positions in an 8.5-kb *Eco*RI fragment and a 2.3-kb *Bam*HI fragment (Fig. 3). The subcloned *Eco*RI and *Bam*HI fragments (pIJ9222 and pIJ9228, respectively) complemented A700 for AHL production. Plasmid pIJ9228 also enabled a non-AHL-producing strain (C58.00) of *Agrobacterium* to make AHLs detected by CV026 (Fig. 1, lane e) or by *A. tumefaciens* carrying *traG-lacZ* as a detection system (Fig. 2, lane f). The pattern of AHLs produced by C58.00/pIJ9228 was similar to that produced by 8401 (Fig. 1 and 2) except that no 3OH,$C_{14:1}$-HSL was detected and one additional fast-migrating spot was detected (Fig. 2, lane f). Occasionally, we have detected such a fast-migrating component in the growth medium supernatant of strain 8401 (data not shown).

The DNA sequence of the 2.3-kb *Bam*HI fragment revealed two genes strongly homologous to *raiI* and *raiR* from *R. etli* (25). Sequencing of various end fragments revealed identities to parts of *dadR*, *dadX*, and *dadA* from *R. leguminosarum* strain 3841 (1) (accession number AJ249196). The deduced map of the region based on whole and partial sequence alignments is shown in Fig. 3. Thus, *raiI* and *raiR* are adjacent to a gene (*dadR*) encoding a regulator in the LRP family, which regulates adjacent genes that encode enzymes involved in alanine metabolism (1). Also shown in Fig. 3 are *orf1*, an incomplete open reading frame (136 amino acids) that shows 37% identity over a 60-amino-acid region with a glucoamylase (accession number P26989) from *Schizosaccharomyces pombe*; a partial open reading frame that shows 81% identity with a predicted (34) aldehyde dehydrogenase (AldA) from *Agrobacterium radiobacter* (accession number X95394); and an open reading frame which shows 52% identity to β-alanine pyruvate transaminase from *Pseudomonas putida* (A42800) and is very similar (72 and 68% identities, respectively) to predicted omega amino transferases (*oatA*) from *S. meliloti* (CAC47872) and *Agrobacterium* (AAK90062). Although the *dadR-oatA* region is highly conserved at the DNA sequence level (about 98% identity) between two isolates (strains 8401 and 3841) of *R. leguminosarum*, the sequence similarity ends abruptly downstream of *dadR*. No sequence corresponding to *raiR* was found in the region downstream of *dadR* sequenced previously from strain 3841 (1). The difference is not due to a cloning artifact, because DNA hybridization failed to detect *raiR* in strain 3841 (data not shown). This is in accord with the previous conclusion that strain 3841 does not produce the AHLs defined here as being made by RaiI (18). This suggests that the *raiIR* gene region has recently been lost by 3841 or has recently been acquired by 8401. The observation that the context of the *raiIR* genes in *R. etli* is similar to that in strain 8401 (25) may imply that a loss from strain 3841 is more likely. Such events may partially explain the diversity of AHLs found in different strains (7, 18).

DNA hybridization revealed that strain 8401 contains a single copy of the *raiIR* locus (data not shown). To determine if the *raiIR* gene region is located on the chromosome or on one of the two large plasmids (of about 400 and 600 kb) in strain 8401, the plasmids were separated electrophoretically and hybridized with a *raiIR* probe. The probe hybridized strongly to

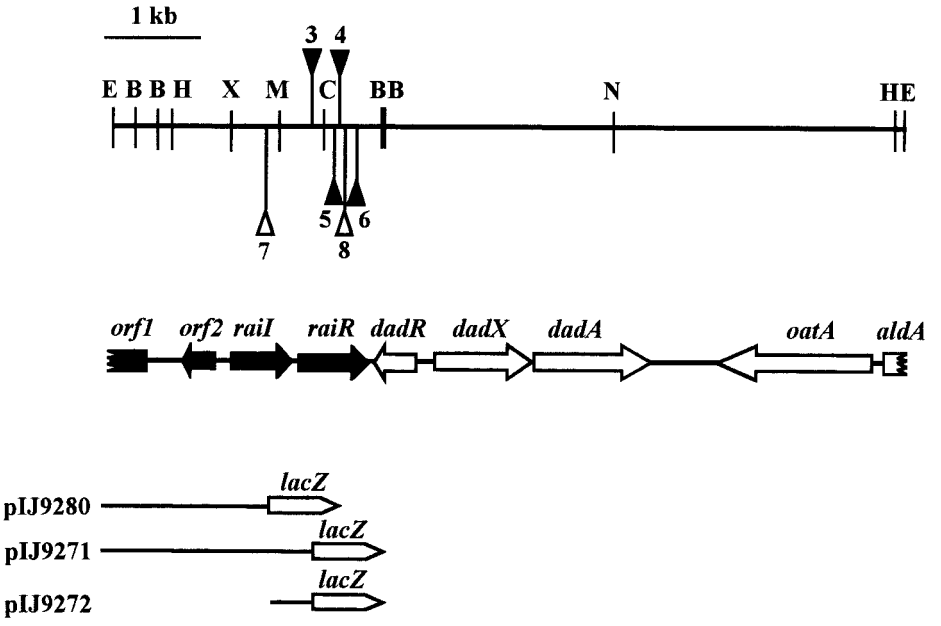

FIG. 3. Map of the *rai* gene region. Open reading frames corresponding to *raiI*, *raiR*, *dadR*, *dadX*, and *dadA* are shown as thick arrows. Filled triangles, locations of the four Tn*5* insertions obtained after mutagenesis of cosmid pIJ9001 (pIJ9161 *raiR5*::Tn*5*, pIJ9163 *raiR3*::Tn*5*, pIJ9164 *raiR4*::Tn*5*, and pIJ9165 *raiR6*::Tn*5*). Open triangles, locations of the *raiI7*::Tn*5* insertion (in plasmids pIJ9231 and pIJ9237 and in mutants A789, A793, A797, and A798) and the *raiR8*::Tn*5* insertion (in plasmids pIJ9234 and pIJ9238 and in mutants A802 and A803). The DNA fragments used to create the *raiI-lacZ* plasmid (pIJ9280), the *raiR-lacZ* plasmid carrying *raiI* (pIJ9271), and the *raiR-lacZ* plasmid that does not carry *raiI* (pIJ9272) are shown. Some restriction enzyme sites are shown, abbreviated as follows: B, *Bam*HI; E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; M, *Mfe*I; N, *Not*I; X, *Xho*I.

the larger of the two plasmids (data not shown). On this basis we conclude that the *raiIR* genes are plasmid located.

The *raiR* gene is predicted to encode a protein that belongs to the LuxR family of transcriptional regulators. *R. leguminosarum* RaiR is 88% identical to RaiR from *R. etli* but showed no such strong similarity with any other LuxR-like gene products in the *S. meliloti* or *M. loti* database. RaiR is 30 and 21% identical, respectively, to CinR and RhiR, two other LuxR-type regulators from *R. leguminosarum* bv. viciae (17, 24). Similar levels of identity (25 to 30%) were found with a range of other LuxR-type regulators such as CerR from *Rhodobacter sphaeroides* (32%; accession number AF16298); TraR from *Rhizobium* sp. strain NGR234 (27%; P55407); RhlR (26%; P54292) and VsmR (25%; U15644), both from *Pseudomonas aeruginosa*; and VanR from *Vibrio anguillarum* (25%; U69677).

The predicted *raiI* gene product belongs to the LuxI family of AHL synthases. RaiI is 93% identical to RaiI from *R. etli* but only 33 and 25% identical, respectively, to CinI and RhiI, two other AHL synthases from *R. leguminosarum* bv. viciae. CinI produces 3OH,$C_{14:1}$-HSL (17), while RhiI produces C_6-HSL, C_8-HSL (24), and another component now known to be C_7-HSL (17). Similar levels of identity (27 to 35%) were seen with the LuxI-like proteins in the *S. meliloti* and *M. loti* databases.

Phenotypes of defined *raiI* and *raiR* mutants. Following recombination and transduction, we transferred the *raiI7*::Tn*5* and *raiR8*::Tn*5* alleles (Fig. 3) into strain 8401 (lacking a symbiosis plasmid) to generate A789 (*raiI7*::Tn*5*) and A802 (*raiR8*::Tn*5*) and into A34 (carrying the symbiosis plasmid pRL1JI) to generate A793 (*raiI7*::Tn*5*) and A803 (*raiR8*::Tn*5*). A789 and A802 produced no AHLs detectable by *C. violaceum* CV026 (Fig. 4, lanes b and c). By use of *A. tumefaciens* carrying *traG-lacZ* as a detection system (Fig. 2), it is evident that the mutation in A789 abolished the production of most of the AHLs (Fig. 2, lane e), and a similar result was seen with A802 (data not shown). As expected, 3OH,$C_{14:1}$-HSL is made normally by A789 (Fig. 2, lane e) and A802 (data not shown), and this was confirmed by bacteriocin type bioassays. A small spot of an unknown component was also detected in the culture supernatant of A789 (Fig. 2, lane e), but since an extract of an uninoculated growth medium also revealed a similar component (Fig. 2, lane g), we conclude that this is not a *Rhizobium*-specific AHL.

To analyze the possible effects of *raiI* and *raiR* mutations on AHL production loci on pRL1JI, A793 (*raiI*) and A803 (*raiR*) were assayed for AHL production by using CV026, which detects various pRL1JI-determined AHLs (17, 24). No significant difference from A34 was seen (Fig. 4). Therefore, we conclude that *raiI* and *raiR* are not required for the production of pRL1JI-determined AHLs.

Strains A34 (wild type), A793 (*raiI*), and A803 (*raiR*) were inoculated onto peas for nodulation tests. The rate of nodulation and number of nodules formed were similar in each case. A793 and A803 consistently nodulated at a slightly higher level than the wild type (10 to 20% more), but this was not statistically significant at the 95% confidence level.

To assess if nitrogen fixation was normal in the nodules formed, the levels of acetylene reduction by the nodulated root system were analyzed. Again, there was no significant difference between A34 and any of the mutants. The nodules formed by the mutants were a healthy pink color typical of nitrogen-fixing nodules, and no signs of nitrogen stress were visually observed in the plant.

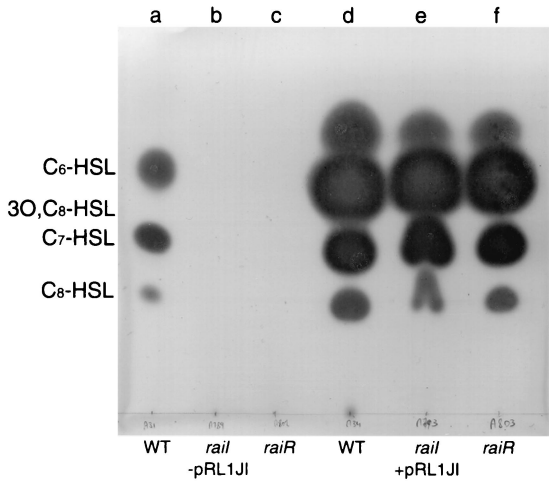

FIG. 4. Mutation of *raiI* or *raiR* does not influence production of AHLs by pRL1JI. Cultures were grown in 100 ml of TY broth. AHLs extracted from cell-free culture supernatants were separated by TLC and detected by using an overlay of agar seeded with *C. violaceum* CV026. Each lane was loaded with a volume of extract equivalent to 25 ml of culture. Lanes a and d, extracts from wild-type *R. leguminosarum* 8401 and A34 (8401/pRL1JI), respectively; lanes b and e, extracts from *raiI7*::Tn*5* mutants A789 (−pRL1JI) and A793 (+pRL1JI), respectively; lanes c and f, extracts from *raiR8*::Tn*5* mutants A802 (−pRL1JI) and A803 (+pRL1JI), respectively. The migration positions of standards (not shown) are indicated to the left of the chromatogram.

Identification of AHLs produced by RaiI. Strain 8401 was previously shown to produce C_6-HSL, C_7-HSL, and C_8-HSL (17, 24), and it is now clear that mutation of *raiI* abolishes the production of these AHLs (Fig. 1). Furthermore, *A. tumefaciens* C58.00 (which produces no AHLs [17, 35]) containing *raiI* cloned in pIJ9228 produced an AHL profile similar to that of 8401 (Fig. 1 and 2), and mutation of *raiI* (pIJ9231) blocked the production of these AHLs (data not shown). Therefore, we conclude that RaiI produces C_6-HSL, C_7-HSL, and C_8-HSL. However, RaiI also made other components (Fig. 2), one of which comigrated with 3O,C_8-HSL (and with 3OH,C_8-HSL [data not shown]). This component was not identified in earlier work, because it was not detected by the *C. violaceum* CV026 detection system used in that work (17, 24).

The band corresponding to this component made in C58.00/pIJ9228 was scraped off a preparative TLC plate. ES-MS revealed two strong peaks of *m*/*z* 244.2 and 266.2, which correspond to the predicted $[M + H]^+$ and $[M + Na]^+$ quasimolecular ions of 3OH,C_8-HSL, and a peak of 101.8, corresponding to the homoserine lactone moiety. In addition, a smaller peak of *m*/*z* 226.2 was observed, and this could correspond to the $[M + H]^+$ of a dehydration product of 3OH,C_8-HSL; such dehydration products are typically observed with hydroxy (but not oxo) homoserine lactones (16, 22, 28). 3OH,C_8-HSL was chemically synthesized, essentially as described previously (8), and when this was used as a standard, peaks of *m*/*z* 244.4, 266.4, 226.2, and 102 were detected. We conclude that this AHL produced by RaiI is 3OH,C_8-HSL, based on the mass spectrum; this is consistent with the migration position and the strong activation of the *Agrobacterium* detection system but weak activation of the *C. violaceum* detection system. ES-MS of fractions corresponding to the two fastest-migrating components detected (Fig. 2, lane f) revealed no major peaks corresponding to predicted *m*/*z* peaks for known AHLs.

To determine the relative levels of the different AHLs produced by RaiI, we separated AHLs produced by strain 8401 by TLC using the same conditions as those for the experiment for which results are shown in Fig. 2. Following chromatography, we spotted onto the dried plate a dilution series of chemically synthesized standards of C_6-HSL, C_7-HSL, C_8-HSL, and 3OH,C_8-HSL. This was done in duplicate, and the plates were then developed with *A. tumefaciens* NT1/pZLR4 or *C. violaceum* CV026. By comparing the relative staining intensities of the diluted standards with those of the AHLs produced by 8401, we estimated that a stationary-phase culture of strain 8401 contains about 0.1 to 0.2 μM 3OH,C_8-HSL (assuming no losses during extraction). The estimated concentrations of C_6-HSL and C_7-HSL were about 1,000-fold lower, while that of C_8-HSL was about 10-fold lower.

R. leguminosarum is rather unusual in that it makes two hydroxy-substituted AHLs, 3OH,$C_{14:1}$-HSL from CinI and 3OH,C_8-HSL from RaiI. Other strains that make hydroxy-substituted AHLs include *Pseudomonas fluorescens* (16, 28), *Vibrio harveyi* (3), and *V. anguillarum* (22). *P. fluorescens* has an HtdS-type AHL synthase (16), and the latter two bacteria use LuxM-type rather than LuxI-type AHL synthases (3, 22), so currently, RaiI and CinI are two of the very few described LuxI-type AHL synthases that make hydroxy-substituted AHLs.

TABLE 2. Effects of added AHLs on *raiI-lacZ* expression[a]

AHL	*raiI-lacZ* (pIJ9280) expression (Miller units)					
	A. tumefaciens C58.00		A789 (*raiI7*::Tn*5*)		A797 (*cinI3*::Spc *raiI7*::Tn*5*)	
	Without *raiR*	With pIJ9276 (*raiR*)	Without *raiR*	With pIJ9276 (*raiR*)	Without *raiR*	With pIJ9276 (*raiR*)
None	140 ± 12	150 ± 14	462 ± 25	1,695 ± 31	326 ± 19	364 ± 15
C_6-HSL	135 ± 11	121 ± 13	336 ± 21	856 ± 11	295 ± 12	353 ± 17
C_7-HSL	139 ± 14	161 ± 16	345 ± 22	1,113 ± 14	289 ± 14	335 ± 16
C_8-HSL	142 ± 15	278 ± 19	331 ± 15	1,542 ± 17	291 ± 17	359 ± 17
3O,C_6-HSL	154 ± 13	297 ± 15	332 ± 12	687 ± 15	317 ± 15	471 ± 15
3O,C_8-HSL	147 ± 11	4,135 ± 27	913 ± 54	7,032 ± 84	617 ± 16	4,550 ± 34
3OH,C_8-HSL	141 ± 10	12,135 ± 119	3,854 ± 49	16,520 ± 164	1,338 ± 23	14,070 ± 157
3OH,$C_{14:1}$-HSL	151 ± 13	1,272 ± 115	493 ± 17	2,094 ± 57	305 ± 21	895 ± 42

[a] AHLs were used at a final concentration of 1 μM, and β-galactosidase activity was assayed after 24 h for *A. tumefaciens* and after 48 h for *R. leguminosarum*.

Assay of AHLs that induce *raiI-lacZ*. In order to assay which added AHLs induce *raiI* expression, a *raiI-lacZ* fusion (pIJ9280) was made (Fig. 3) and was introduced into *A. tumefaciens* C58.00, which makes no AHLs. In the absence of *raiR*, only background levels of activity were seen and the addition of various AHLs had no effect (Table 2). The results of an assay of *raiI-lacZ* expression with *raiR* present are also shown in Table 2. In the absence of added AHLs, *raiR* (on pIJ9276) had no effect on expression compared with the background level seen when *raiR* was absent. Various AHLs were tested for *raiR*-dependent induction of *raiI-lacZ* expression in *A. tumefaciens* C58.00/pIJ9280. The strongest induction was seen with 3OH,C_8-HSL, and lower levels were seen with 3O,C_8-HSL and 3OH,$C_{14:1}$-HSL. No induction was seen with C_6-HSL, C_7-HSL, C_8-HSL, or 3O,C_6-HSL (Table 2). These results demonstrate that *raiI* is regulated by RaiR and that 3OH,C_8-HSL, a product of RaiI, is the strongest inducer identified (Table 2). However, it is evident that some AHLs made at a low level by RaiI (C_6-HSL, C_7-HSL, and C_8-HSL) do not induce much *raiI-lacZ* expression (Table 2). Paradoxically, the data also suggest that AHLs made by other gene products such as CinI can induce *raiR*-dependent *raiI* expression (Table 2).

The assay of *raiI-lacZ* (pIJ9280) in the *R. leguminosarum raiI* mutant (A789) is complicated by the observation (see below) that the Tn*5* in *raiI* may affect the expression of *raiR,* so the assays were done in the absence and in the presence (on pIJ9276) of *raiR*. Only a low level of activity was seen in A789 (*raiI*) carrying *raiI-lacZ* on pIJ9280, and 3OH,C_8-HSL induced the most expression, although 3O,C_8-HSL also gave some induction (Table 2). Cloned *raiR* on pIJ9276 resulted in significant *raiI-lacZ* expression in A789, even though no AHLs were added. This suggested that either CinI-made 3OH,$C_{14:1}$-HSL may induce *raiI* or multicopy *raiR* induced AHL-independent *raiI* expression. To test this, pIJ9280 (*raiI-lacZ*) was transferred to a *raiI cinI* double mutant of *R. leguminosarum* (A797). Mutation of *cinI* in addition to *raiI* greatly reduced *raiI-lacZ* expression in the presence of cloned *raiR* (Table 2). The decrease in expression of *raiI-lacZ* in the *raiI cinI* mutant compared with the *raiI* mutant indicates that CinI-made AHLs are responsible for the expression of *raiI-lacZ* in the *raiI* mutant A789 (Table 2). This is consistent with the observation that cloned *raiR* induced no expression of *raiI-lacZ* in the absence of added AHLs in *A. tumefaciens* C58.00 (Table 2). The effects of AHLs on *raiI-lacZ* expression in A797 (*raiI cinI* mutant) carrying cloned *raiR* are broadly similar to the results seen with AHLs added to *A. tumefaciens* carrying *raiI-lacZ* (Table 2). In the absence of cloned *raiR*, in A797 (*raiI cinI* mutant), 3OH,C_8-HSL induced *raiI-lacZ* expression most strongly (Table 2); the level of expression was lower than that induced by 3OH,C_8-HSL in A789 (*raiI* mutant), implying a role for CinI-made 3OH,$C_{14:1}$-HSL in the higher expression seen in A789 (*raiI* mutant) than in A797 (*rai cinI* mutant).

The background level of *raiI-lacZ* expression seen in the *raiI* mutant (A789) carrying cloned *raiR* was decreased by addition of C_6-HSL, C_7-HSL, or 3O,C_6-HSL (Table 2). This implies that these AHLs may compete with 3OH,C_{14}-HSL for the binding site on RaiR. At lower levels (20 nM), 3OH,C_8-HSL and 3O,C_8-HSL induced *raiI-lacZ* expression in *A. tumefaciens* C58.00/pIJ9280 (9,324 ± 210 and 1,350 ± 33 Miller units, respectively) but 3OH,$C_{14:1}$-HSL did not (160 ± 14 Miller units), suggesting that lower threshold concentrations of 3OH,C_8-HSL and 3O,C_8-HSL may be required for activation of *raiI* induction by RaiR.

TABLE 3. Genetic complementation of *raiI* and *raiR* mutants[a]

Plasmid	AHL production[b] detected by CV026 in			
	8401	A789 *raiI7*::Tn*5*	A802 *raiR8*::Tn*5*	A700
None	+	−	−	−
pIJ9228 (*raiI raiR*)	+++	+++	+++	+++
pIJ9231 (*raiI7*::Tn*5 raiR*)	+	−	+	−
pIJ9234 (*raiI raiR8*::Tn*5*)	+	+/−	−	+/−

[a] AHL production was tested on a plate assay using *C. violaceum* CV026 as the indicator strain.

[b] −, no complementation; +/−, weak complementation (less than 10% of AHL levels produced by 8401); +, complementation; +++, strong complementation (the AHL production level was greater than that of the control 8401).

Complementation tests with *raiI* and *raiR* mutants. Genetic complementation tests were carried out with plasmids containing the cloned *raiIR* genes and derivatives of these plasmids carrying Tn*5* insertions in *raiI* or *raiR*. These plasmids were introduced into A789 (*raiI*) and A802 (*raiR*), and AHLs were measured. Similar results were obtained using the *C. violaceum* CV026 and *A. tumefaciens* NT1/pZLR4 systems. Introduction of the cloned *raiIR* genes on pIJ9228 into 8401 resulted in enhanced AHL production (Table 3); we estimate that the overall level was about 5- to 10-fold higher, and mutation of *raiI* or *raiR* on the introduced plasmid (pIJ9231 or pIJ9234) reduced AHL production to wild-type levels. In the presence of pIJ9228, mutation of the genomic copy of *raiI* (A789) or *raiR* (A802) had relatively little effect on AHL production.

There was essentially no (or very low levels of) production of AHLs by cloned *raiI* in the absence of *raiR*, as seen with A802/pIJ9234 (Table 3). The observation (Table 3) that A789 (*raiI7*::Tn*5*) was very poorly complemented (AHL levels were less than 10% of that seen in the 8401 control) by pIJ9234 (carrying *raiI* and *raiR*::Tn*5*) indicates that the *raiI* mutation in A789 may have a polar effect on *raiR.* When the *raiI*7::Tn*5* mutation was on the plasmid (pIJ9231) in the *raiR* mutant A802, the polarity was less evident. This may have been due to the multicopy plasmid allowing expression of *raiR*.

We analyzed the complementation characteristics of the original mutant A700. There was weak complementation with pIJ9234 (carrying *raiI* and a mutation in *raiR)* but no complementation with pIJ9231, carrying a mutation in *raiI*. Therefore, A700 probably carries a mutation in *raiI*. Genomic DNA was hybridized with a *raiIR* probe, and this revealed that there was an insert of about 1.7 kb in the *raiI* region (data not shown). Given that the insertion is around 1.7 kb, possibly an IS*50* element may have transposed separately from the Tn*5-gus* transposon used for the mutagenesis. Similar events have been observed previously in *R. leguminosarum* (19).

***raiI* is positively autoregulated by RaiR.** Analysis of β-galactosidase activity with 8401/pIJ9280 (*raiI-lacZ*) revealed that *raiI* is upregulated as the cells enter the late-exponential phase of growth. Mutation of *raiI* or *raiR* in strain 8401 blocked induction of *raiI-lacZ* (Fig. 5). When pRL1JI was present (A34), *raiI-lacZ* expression was similar to that seen with 8401, except that the induction was slightly earlier (Fig. 5). This effect was consistent in different tests, and we did not observe

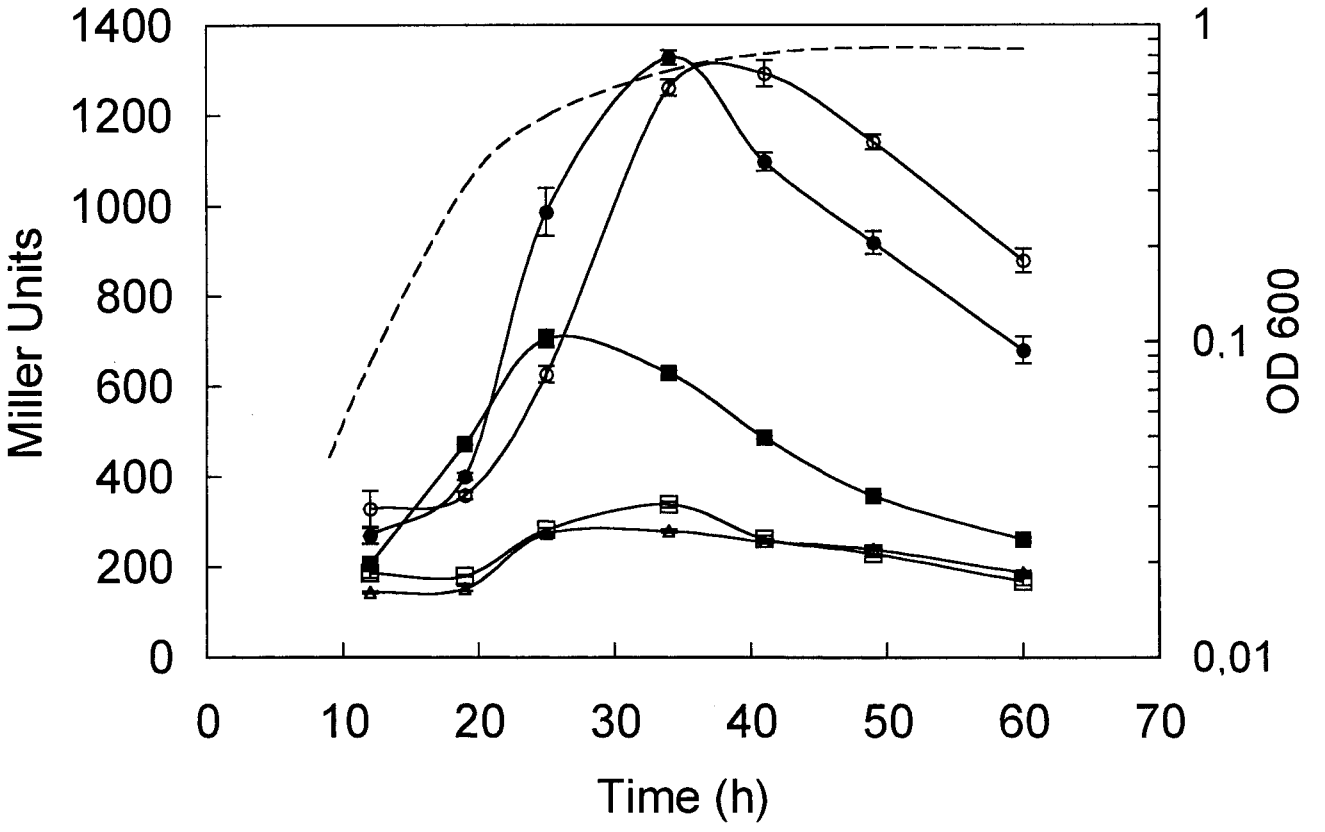

FIG. 5. Expression of *raiI-lacZ*. The expression of *raiI-lacZ* (pIJ9280) was analyzed by measuring β-galactosidase levels throughout the growth of strains 8401 (open circles), 8401/pRL1JI (solid circles), A789 (8401 *raiI7*::Tn*5*) (open squares), A793 (8401/pRL1JI *raiI7*::Tn*5*) (solid squares), and A802 (8401 *raiR8*::Tn*5*) (open triangles). The growth curve (OD_{600}) shown as a broken line corresponds to that obtained with 8401/pIJ9280; the growth of the other strains was very similar.

any differences between the growth of A31 and that of A34. In the presence of pRL1JI, mutation of *raiI* (A793) did not block *raiI-lacZ* expression, although the level of induction was lower than that seen in the control, A34 (Fig. 5). These observations are consistent with the hypothesis that *raiI* is positively autoregulated (by AHLs made by RaiI) but that pRL1JI may determine the production of AHLs which can also induce *raiI-lacZ*. As shown above (Table 2), the *rhiI*-made AHLs (C_6-HSL, C_7-HSL, and C_8-HSL) are unlikely to be responsible for this activation. In other work (V. Danino, A. Wilkinson, and J. A. Downie, unpublished data) we have established that the other AHL synthase (*traI*) on pRL1JI produces AHLs that activate *raiR*-dependent *raiI-lacZ* expression.

Two other *lacZ* fusion plasmids (Fig. 3), pIJ9271 (carrying *raiI raiR-lacZ*) and pIJ9272 (carrying *raiR-lacZ* but lacking *raiI* and the predicted *raiI* promoter region), were constructed. The *raiR-lacZ* fusion (pIJ9272) was expressed in strain 8401 at a low level (330 ± 20 Miller units), indicating that either *raiR* does not have a separate promoter or, if it does, it has a relatively weak one. The level of *raiR-lacZ* expression in the *raiR* mutant (A802) was similar (295 ± 17 Miller units), indicating that there is not a RaiR-regulated promoter between *raiI* and *raiR*. The *raiIR-lacZ* fusion (pIJ9271) was expressed at a slightly higher level (395 ± 20 Miller units) in 8401, and this was decreased in the *raiR* mutant (320 ± 20 Miller units). These results indicate that *raiR* may be expressed at a low level that is independent of the *raiI* promoter, but as *raiI* expression is induced, so is that of *raiR*, such that the *raiIR* genes may be positively autoregulated (by RaiR). In strain 8401, there was significantly less expression of *raiIR-lacZ* (395 ± 20 Miller units) than of *raiI-lacZ* (1,261 ± 28 Miller units), suggesting that there may be some kind of transcriptional attenuation between *raiI* and *raiR*. The 90-bp region between *raiI* and *raiR* was examined for possible secondary structures. A region of dyad symmetry was found flanking either side of the predicted

TABLE 4. Effect of *raiR* on *raiI-lacZ* expression[a]

Strain	*raiI-lacZ* (pIJ9280) expression		
	Alone	+ 3OH,C8-HSL	+ *raiR* (pIJ9276)
8401	1,261 ± 18	4,640 ± 29	21,517 ± 340
A789 *raiI7*::Tn*5*	462 ± 16	3,854 ± 31	1,695 ± 31
A802 *raiR8*::Tn*5*	344 ± 13	299 ± 11	18,606 ± 278
A643 *cinI3*::Spc	346 ± 18	1,330 ± 19	16,222 ± 271
A552 *cinR1*::Spc	364 ± 17	1,738 ± 53	14,194 ± 205
A797 *cinI3*::Spc *raiI7*::Tn*5*	326 ± 13	1,338 ± 19	364 ± 15

[a] β-Galactosidase activity was assayed after 24 h of growth and is expressed in Miller units (21) ± standard errors.

translation start of RaiR. This may influence the level of *raiR* expression.

Adding *raiR* (on pIJ9276) to the control strain (8401) greatly increased the level of *raiI-lacZ* expression (Table 4). This indicates that the availability of RaiR is a limiting factor in *raiI* expression. The cloned *raiR* gene also increased the expression of the *raiIR-lacZ* fusion in 8401 (from 395 ± 20 to 1,200 ± 37 Miller units) but not that of the *raiR-lacZ* fusion, which remained constant (330 ± 30 Miller units). This result confirms that expression of *raiR* is influenced by the *raiI* promoter; however, the low-level constitutive expression of *raiR* was unaffected by cloned *raiR*.

***raiI* expression is controlled by the *cinRI* locus.** We previously observed that mutation of *cinI* in 8401 greatly reduces the production of AHLs detected by *C. violaceum* CV026 (17). This implies that the *cinRI* locus influences *raiI* expression. This was tested by transferring *raiI-lacZ* (pIJ9280) into *cinI* and *cinR* mutant derivatives of 8401. As shown (Table 4), both of these genes are required for normal expression of *raiI*. Although mutation of *raiI* strongly reduced *raiI-lacZ* expression, this expression was further reduced (from 462 to 326 Miller units) in the *rai cinI* double mutant A797 (Table 4). This could be consistent with the hypothesis that the *raiI* promoter may respond to the CinI-made 3OH,$C_{14:1}$-HSL in addition to RaiI-made AHLs. However, added 3OH,C_8-HSL only partially suppressed the effects of mutations in *cinI* or *cinR* on *raiI-lacZ* expression (Table 4). This may imply that *cinI* and *cinR* have an indirect effect on the expression of *raiI*.

Cloned *raiR* almost completely suppressed the effects of mutating *cinI* or *cinR* (Table 4). In a *raiI* mutant background, no such strong suppression was seen, demonstrating that RaiI-made AHLs are required for this effect. There is expression of *raiI-lacZ* in the *raiI* mutant carrying cloned *raiR* (1,695 Miller units), and this is probably due to CinI-made 3OH,$C_{14:1}$-HSL, because the level of expression is reduced (to 364 Miller units) in the *cinI raiI* double mutant A797 (Table 4). One explanation for these observations is that the *raiIR* genes are initially induced in response to CinI-made 3OH,$C_{14:1}$-HSL, and then as RaiI-made AHLs accumulate, expression of *raiI* is autoinduced. In support of this model, we have consistently observed a delay (of about 2 to 3 h) in the induction of *raiI-lacZ* expression in strain 8401 compared with assays of *cinI-lacZ* described previously (17). The timing of *raiI* induction is somewhat earlier in growth if pRL1JI is present (Fig. 5), indicating that AHLs determined by pRL1JI (*traI*) could also stimulate *raiI* expression.

Conclusions. It is evident that the *raiIR* genes constitute part of a quorum-sensing network in *R. leguminosarum* and probably also in *R. etli* (25). The next step is to identify the genes regulated by RaiR in response to RaiI-made AHLs. The types of genes regulated are likely to play some sort of role in environmental adaptation that is not readily seen in laboratory tests of growth or nodulation. The observation that the *raiIR* genes may be absent from some strains of *R. leguminosarum* points toward a potentially subtle role for these genes. Recent comparisons of the plasmid and genome sequences of *Sinorhizobium* strains have revealed that many genes present in one strain are absent from another (11). Identifying the roles of such genes may give insights into aspects of subtle but specific interactions between rhizobia and their host plants and environment.

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ADDENDUM IN PROOF

After submission of this work, R. S. Blosser-Middleton and K. M. Gray (J. Bacteriol. **183:**6771–6777, 2001) described an analysis of [α-^{14}C]methionine-labeled AHLs made by strains of *R. leguminosarum* used here also. In their work, only comigrating standards were used to identify the AHLs made by strains carrying or lacking pRL1JI. They assumed that 3O,C_8-HSL was being made by both strains and concluded that the kinetics of production of this AHL was different depending on whether pRL1JI was present or absent. A lower rate of formation was thought to occur when pRL1JI was absent. However, the chemical characterization of 3OH,C_8-HSL in the work described here means that the data of Blosser-Middleton and Gray should be reinterpreted. It is evident that 3O,C_8-HSL and 3OH,C_8-HSL comigrate, so some of their conclusions are probably incorrect. We propose that the different kinetics of appearance of the AHL that comigrates with 3O,C_8-HSL is due to the formation of two products, one being 3OH,C_8-HSL made by RaiI and one being 3O,C_8-HSL made by TraI. In the strain carrying pRL1JI, we detected both 3OH,C_8-HSL and 3O,C_8-HSL, but when pRL1JI (and hence *traI*) was absent, no 3O,C_8-HSL was detected. The slower induction of *raiI-lacZ* (and hence the appearance of 3OH,C_8-HSL) compared to that of *cinI-lacZ* (Fig. 5) is consistent with delayed production of 3OH,C_8-HSL during growth of the strain lacking pRL1JI. There are several other aspects of the analysis of AHL formation as described by Blosser-Middleton and Gray that need to be reassessed in light of the work described here.

REFERENCES

1. **Allaway, D., E. M. Lodwig, L. A. Crompton, M. Wood, R. Parsons, T. R. Wheeler, and P. S. Poole.** 2000. Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Mol. Microbiol. **36:**508–515.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. **13:**273–286.
4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188–198.
5. **Beynon, J. L., J. E. Beringer, and A. W. B. Johnston.** 1980. Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. J. Gen. Microbiol. **120:**421–429.
6. **Buchanan-Wollaston, A. V.** 1979. Generalised transduction in *Rhizobium leguminosarum*. J. Gen. Microbiol. **112:**135–142.
7. **Cha, C., P. Gao, Y.-C. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119–1129.
8. **Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, G. S. A. B.**

Stewart, and B. J. Bycroft. 1993. Autoregulation of carbapenem antibiotic synthesis in *Erwinia carotovora* ATCC 39048 by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J. Antibiot. **46:**441–454.

9. **Cubo, M. T., A. Economou, G. Murphy, A. W. B. Johnston, and J. A. Downie.** 1992. Molecular characterisation and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar viciae. J. Bacteriol. **174:**4026–4035.
10. **Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen.** 1985. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol. Gen. Genet. **198:**255–262.
11. **Galibert, F., T. Finan, S. R. Long, A. Puhler, P. Abola, F. Ampe, et al.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.
12. **Gray, K. M., J. P. Pearson, J. A. Downie, B. E. A. Boboye, and E. P. Greenberg.** 1996. Cell-to-cell signalling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. J. Bacteriol. **178:**372–376.
13. **Hirsch, P. R.** 1979. Plasmid-determined bacteriocin production by *Rhizobium leguminosarum.* J. Gen. Microbiol. **113:**219–228.
14. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
15. **Lamb, J. W., G. Hombrecher, and A. W. B. Johnston.** 1982. Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli.*Mol. Gen. Genet. **186:**449–452.
16. **Laue, B. E., Y. Jiang, S. R. Chhabra, S. Jacob, G. S. A. B. Stewart, A. Hardman, J. A. Downie, F. O'Gara, and P. Williams.** 2000. The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium* small bacteriocin, *N*-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology **146:**2469–2480.
17. **Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dyé, P. Williams, and J. A. Downie.** 2000. The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. Mol. Microbiol. **37:**81–97.
18. **Lithgow, J. K., V. E. Danino, J. Jones, and J. A. Downie.** 2001. Analysis of *N*-acyl homoserine-lactone quorum-sensing molecules made by different strains and biovars of *Rhizobium leguminosarum* containing different symbiotic plasmids. Plant Soil **232:**3–12.
19. **Mavridou, A., M.-A. Barny, P. Poole, K. Plaskitt, A. E. Davies, A. W. B. Johnston, and J. A. Downie.** 1995. *Rhizobium leguminosarum* nodulation gene (*nod*) expression is lowered by an allele-specific mutation in the dicarboxylate transport gene *dctB*. Microbiology **141:**103–111.
20. **McLean, K. H., M. K. Winson, L. Fish, A. Taylor, S. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.
21. **Miller, J. A.** 1976. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Milton, B. L., V. J. Chalker, D. Kirke, A. Hardman, M. Camara, and P. Williams.** 2001. The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone. J. Bacteriol. **183:**3537–3547.
23. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram negative bacteria. Gene **127:**15–21.
24. **Rodelas, B., J. K. Lithgow, F. Wisniewski-Dyé, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. viciae. J. Bacteriol. **181:**3816–3823.
25. **Rosemeyer, V., J. Michiels, C. Verreth, and J. Vanderleyden.** 1998. *luxI*- and *luxR*-homologous genes of *Rhizobium etli* CNPAF512 contribute to synthesis of autoinducer molecules and nodulation in *Phaseolus vulgaris*. J. Bacteriol. **180:**815–821.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Sharma, S. B., and E. Signer.** 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn*5-gusA*. Genes Dev. **4:**344–356.
28. **Shaw, P. D., G. Ping, S. L. Daly, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036–6041.
29. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilisation of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
30. **Sourjik, V., P. Muschler, B. Scharf, and R. Schmitt.** 2000. VisN and VisR are global regulators of chemotaxis, flagellar, and motility genes in *Sinorhizobium (Rhizobium) meliloti.* J. Bacteriol. **182:**782–788.
31. **Spaink, H. P., J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9:**27–39.
32. **Swift, S., J. A. Downie, N. A. Whitehead, A. M. L. Barnard, G. P. C. Salmond, and P. Williams.** 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv. Microb. Physiol. **45:**99–270.
33. **Thorne, S. H., and H. D. Williams.** 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. phaseoli: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. J. Bacteriol. **181:**981–990.
34. **Tibertius, A., N. G. de Luca, H. Hussain, and A. W. B. Johnston.** 1996. Expression of the *exoY* gene, required for exopolysaccharide synthesis in *Agrobacterium*, is activated by the regulatory *ros* gene. Microbiology **142:**2621–2629.
35. **Vaudequin-Dransart, V., A. Petit, C. Poncet, C. Ponsonnet, X. Nesme, J. B. Jones, H. Bouzar, W. S. Chilton, and Y. Dessaux.** 1995. Novel Ti plasmids in *Agrobacterium* strains isolated from fig tree and chrysanthemum tumors and their opinelike molecules. Phytopathology **8:**311–321.
36. **Wheatcroft, R., D. G. McRae, and R. W. Miller.** 1990. Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development. Mol. Plant-Microbe Interact. **3:**9–17.
37. **Wilkinson, A.** 1998. Acyl-homoserine lactone signalling in *Rhizobium leguminosarum*. Ph.D. thesis. University of East Anglia, Norwich, United Kingdom.
38. **Williams, P., T. Baldwin, and J. A. Downie.** 1999. Bacterial crosstalk--communication between bacteria, plant and animal cells. Soc. Gen. Microbiol. Symp. **57:**1–35.
39. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Ladzunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**9427–9431.