

## Analysis of Quorum-Sensing-Dependent Control of Rhizosphere-Expressed (*rhi*) Genes in *Rhizobium leguminosarum* bv. *viciae*

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The *rhi* genes of *Rhizobium leguminosarum* biovar *viciae* are expressed in the rhizosphere and play a role in the interaction with legumes, such as the pea. Previously (K. M. Gray, J. P. Pearson, J. A. Downie, B. E. A. Boboye, and E. P. Greenberg, *J. Bacteriol.* 178:372–376, 1996) the *rhiABC* operon had been shown to be regulated by RhiR and to be induced by added *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (3OH,C<sub>14:1</sub>-HSL). Mutagenesis of a cosmid carrying the *rhiABC* and *rhiR* gene region identified a gene (*rhiI*) that affects the level of *rhiA* expression. Mutation of *rhiI* slightly increased the number of nodules formed on the pea. The *rhiI* gene is (like *rhiA*) regulated by *rhiR* in a cell density-dependent manner. RhiI is similar to LuxI and other proteins involved in the synthesis of *N*-acyl-homoserine lactones (AHLs). Chemical analyses of spent culture supernatants demonstrated that RhiI produces *N*-(hexanoyl)-L-homoserine lactone (C<sub>6</sub>-HSL) and *N*-(octanoyl)-L-homoserine lactone (C<sub>8</sub>-HSL). Both of these AHLs induced *rhiA-lacZ* and *rhiI-lacZ* expression on plasmids introduced into an *Agrobacterium* strain that produces no AHLs, showing that *rhiI* is positively regulated by autoinduction. However, in this system no induction of *rhiA* or *rhiI* with 3OH,C<sub>14:1</sub>-HSL was observed. Analysis of the spent culture supernatant of the wild-type *R. leguminosarum* bv. *viciae* revealed that at least seven different AHLs are made. Mutation of *rhiI* decreased the amounts of C<sub>6</sub>-HSL and C<sub>8</sub>-HSL but did not block their formation, and in this background the *rhiI* mutation did not significantly affect the expression levels of the *rhiI* gene or *rhiABC* genes or the accumulation of RhiA protein. These observations suggest that there are additional loci involved in AHL production in *R. leguminosarum* bv. *viciae* and that they affect *rhiI* and *rhiABC* expression. We postulate that the previously observed induction of *rhiA* by 3OH,C<sub>14:1</sub>-HSL may be due to an indirect effect caused by induction of other AHL production loci.

One of the most abundant proteins made by strains of *Rhizobium leguminosarum* bv. *viciae* is the *rhiA* gene product, which was first observed as a heavily stained band following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of stationary-phase cultures (7). *rhiA* is in a three-gene operon (*rhiABC*) that is under the regulatory control of the *rhiR* gene, which encodes a LuxR-type regulator. The *rhiABC-rhiR* gene cluster is located between genes involved in nitrogen fixation (*nifHDK*) and nodulation (*nod*) on the symbiotic plasmid pRL1JI (5). DNA hybridizations have shown that the *rhi* genes are adjacent to nodulation genes in other strains of *R. leguminosarum* bv. *viciae* (9a, 18). Analysis of several strains of *R. leguminosarum* (biovars *viciae*, *trifolii*, and *phaseoli*), *Rhizobium meliloti*, and *Rhizobium* sp. strain NGR234, using antibody to RhiA, showed that RhiA seems to be specific to *R. leguminosarum* bv. *viciae* (6, 7), indicating that it may play some host-specific role in the interaction between

this biovar and its symbiotic partners (pea, vetch, lentil, and *Lathyrus* spp.).

Initially, no effect on nodulation or symbiotic nitrogen fixation was observed for bacteria containing transposon insertions in *rhiA* (7). Subsequently, it was found that mutation of *rhiA* could affect nodulation in some mutant strains that were already impaired for nodulation due to deletion of some of the nodulation genes (5). In particular, in the absence of the *nodFEL* genes, mutations of *rhiA* could be seen to decrease the low level of nodulation even further.

Analysis of *rhiA-lacZ* and *rhiC-phoA* gene fusions revealed that the *rhiABC* genes are strongly induced during the transition from late exponential to early stationary growth phase (15). The *N*-acyl-homoserine lactone (AHL) termed *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (referred to hereafter as 3OH,C<sub>14:1</sub>-HSL) was identified as both an inducer of the *rhiABC* genes and a potent inhibitor of the growth of some strains of *R. leguminosarum* bv. *viciae* (15). Indeed the compound previously known as “small bacteriocin” (17, 35) had been purified and shown to be 3OH,C<sub>14:1</sub>-HSL (30). It was suggested that this AHL induces stationary phase in *R. leguminosarum* bv. *viciae* since it is thought to induce gene expression that inhibits growth but does not kill the cells (15).

Many strains of the *R. leguminosarum* biovars *viciae*, *trifolii*, and *phaseoli* (15, 17, 35, 37) make small bacteriocin (and thus probably make 3OH,C<sub>14:1</sub>-HSL) as does *Rhizobium etli* (26), whereas *R. meliloti* and the closely related *Agrobacterium tumefaciens* do not (17, 37). In addition, *R. etli* makes at least six

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other compounds that are probably AHLs (26). Mutation of one gene, *rail*, in *R. etli* abolished the production of some autoinducers by *R. etli*, but the production of 3OH,C<sub>14:1</sub>-HSL was unaffected (26).

*Rhizobium* strains often contain multiple plasmids, and it is possible that different plasmids encode the production of different AHLs. In this regard it is not yet known if the *small* bacteriocin locus is located on the chromosome or on a plasmid in those strains which make *small* bacteriocin. The locus encoding production of *small* bacteriocin had been shown not to be located on the symbiotic plasmid pRL1JI (17, 37); nevertheless, 3OH,C<sub>14:1</sub>-HSL induces *rhiABC* gene expression. The regulation of the *rhiABC* genes is also affected by genes other than *rhiR* present on the symbiotic plasmid pRL1JI. It was observed (5, 10) that flavonoid inducers of *nod* gene expression decreased (by about 50%) the level of expression of the *rhiABC* operon and that this required the *nod* gene regulator NodD (5, 10). Furthermore, using a plasmid carrying a *rhiA-lacZ* fusion, Gray et al. (15) observed that an ethyl acetate extract containing 3OH,C<sub>14:1</sub>-HSL had different effects on *rhiA* gene expression in strains containing or lacking pRL1JI.

We wish to understand the physiological role of the *rhi* gene region. Analysis of RhiA protein with antiserum revealed that *rhiA* is expressed by bacteria in the rhizosphere but not by nitrogen-fixing bacteria in nodules (7). Database searches revealed no protein sequences with strong similarity to RhiA, RhiB, or RhiC, although it has been shown that RhiC has an N-terminal signal sequence that targets it to the periplasm (5). In this work we have further characterized the *rhi* gene region, identifying an AHL production locus that is involved in the induction of *rhiABC* expression.

#### MATERIALS AND METHODS

**Microbiological techniques.** *Rhizobium* strains were grown in TY medium (2). Antibiotics were added as appropriate to maintain selection for plasmids. Bacterial growth was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) by using an MSE Spectrophotometer. β-Galactosidase activities were measured as described previously (27) by using a Titertek Multiscan Plus spectrophotometer (EFLAB). For measurements of β-galactosidase throughout growth, bacteria from 24-h cultures were diluted 1 in 200 to a starting OD<sub>600</sub> of about 0.002. When added, AHLs were added at the start of growth to a final concentration of 0.1 μM. The AHLs *N*-hexanoyl-L-homoserine lactone (C<sub>6</sub>-HSL),

*N*-(3-oxohexanoyl)-L-homoserine lactone (3O,C<sub>6</sub>-HSL), *N*-octanoyl-L-homoserine lactone (C<sub>8</sub>-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (3O,C<sub>8</sub>-HSL), *N*-(3-oxobutanoyl)-L-homoserine lactone (3O,C<sub>4</sub>-HSL), and *N*-(3-hydroxy-tetradecenoyl)-L-homoserine lactone (3OH,C<sub>14:1</sub>-HSL) were synthesized (4) and kindly provided by Ram Chhabra, School of Pharmaceutical Sciences, University of Nottingham. Nodulation tests were done by using variety Frisson peas (*Pisum sativum* L.) as described previously (8, 23), with a minimum of 12 matched plants per test; two separate tests were carried out with similar results.

**Bacterial strains and plasmids.** *R. leguminosarum* sp. strain 8401 lacks a symbiotic plasmid, and all *Rhizobium* strains used are based on 8401 (Table 1). A34 is a derivative of 8401 carrying the symbiotic plasmid pRL1JI. Strains A160 and A161, which are isogenic with A34, were made by conjugating derivatives of pRL1JI carrying *rhiR1*::Tn5 and *rhiA4*::Tn5 (7) into 8401. The *rhiI5*::Tn5 allele from pIJ7790 (see below) was recombined onto pRL1JI by marker exchange by using pPH1JI to select for recombinants (28). The derivative of pRL1JI carrying *rhiI5*::Tn5 was conjugated into 8401 to form A721, which was confirmed to lack pPH1JI which transfers at a relatively low frequency.

Mutagenesis of pIJ1089 with Tn5 and Tn3HoHo1 was done as described previously (9, 10). Derivatives of pIJ1089 were transferred to strain 8401 and screened for reduced RhiA production by SDS-PAGE analysis of proteins released from cells, following solubilization of proteins in SDS gel loading buffer, as described previously (10). Mutated plasmids were transferred to the *Escherichia coli* strain DH5α by transformation with DNA isolated from the *Rhizobium* strains. The location of Tn3HoHo1 within *rhiI* in pIJ1696 was determined by restriction enzyme mapping. The location of Tn5 within *rhiI* in pIJ7790 was determined by subcloning part of the Tn5 plus flanking DNA as a 4.9-kb *EcoRI*-*BamHI* fragment in pUC19 and sequencing the DNA by using a Tn5-specific primer.

Plasmid pIJ7794 was made by cloning the *rhiI* gene on a 2.5-kb *HindIII*-*SmaI* fragment subcloned from pIJ1089; the fragment was subcloned in pIJ1891, which

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source
<b>Strain</b>		
<i>R. leguminosarum</i>		
8401	Strain lacking a symbiotic plasmid	8
A34	Derivative of 8401 carrying pRL1JI (previously called 8401 pRL1JI)	9
A160	Derivative of A34 carrying <i>rhiR1</i> ::Tn5	5
A161	Derivative of A34 carrying <i>rhiA4</i> ::Tn5	This work
A721	Derivative of A34 carrying <i>rhiI5</i> ::Tn5	This work
<i>A. tumefaciens</i>		
C58.00	Strain lacking the AT and Ti plasmids; does not make detectable AHLs	36
<b>Plasmids</b>		
pRL1JI	<i>R. leguminosarum</i> bv. viciae native symbiotic plasmid	19
pIJ1089	Cosmid carrying 30 kb of pRL1JI, including the <i>nifHD</i> , <i>rhiABCR</i> , and <i>nodOTNMLEFDABCIJ</i> genes	9
pIJ1242	Derivative of pIJ1089 carrying <i>rhiR1</i> ::Tn5	7
pIJ1243	Derivative of pIJ1089 carrying <i>rhiA4</i> ::Tn5	7
pIJ1642	Derivative of pIJ1089 carrying <i>rhiA5</i> ::Tn3HoHo1 ( <i>rhiA-lacZ</i> )	10
pIJ1696	Derivative of pIJ1089 carrying <i>rhiI7</i> ::Tn3HoHo1 ( <i>rhiI-lacZ</i> )	This work
pIJ1769	<i>rhiA-lacZ</i> fusion	5
pIJ1891	Cloning vector derived from pLAFR3	21
pIJ7790	Derivative of pIJ1089 carrying <i>rhiI5</i> ::Tn5	This work
pIJ7794	2.5-kb <i>HindIII</i> - <i>SmaI</i> fragment carrying <i>rhiI</i> in pIJ1891	This work
pIJ7982	<i>rhiI-lacZ</i> fusion in pMP220	This work
pMP220	<i>lacZ</i> promoter probe vector	32

had been first cut with *BamHI*, filled in with Klenow fragment of DNA polymerase, and finally digested with *HindIII*. The *rhiI-lacZ* gene fusion on pIJ7982 was made by cloning a 0.7-kb fragment carrying part of *rhiI* plus the 0.3-kb upstream region into pMP220. The 0.7-kb fragment was subcloned from one of the plasmids, generated by *ExoIII* nuclease deletion, for DNA sequencing.

**Molecular biology techniques.** DNA cloning, ligations, transformation, restriction enzyme mapping, and DNA hybridization were done by standard methods (29). The DNA sequence of *rhiI* was determined on both strands by using an ordered series of *ExoIII*-generated deleted derivatives of the *rhiI* gene region that had been cloned as a blunt-ended *HindIII*-*SmaI* fragment in both orientations in the *HincII* site of pUC19. The sequencing reactions were carried out by using the Amersham Thermosequencing kit and an Applied Biosystems automated sequencer (ABI 377). Database searches of the protein sequence were done by using the BLAST and FASTA (1) programs to find related sequences in the EMBL and SwissProt protein sequence databases.

**Analysis of proteins.** *Rhizobium* strains were grown to an OD<sub>600</sub> of 1.2 in 100 ml of TY medium, and the cells were harvested by centrifugation. The washed cells from 10 ml of culture were resuspended in 1 ml of 0.1 M Tris HCl (pH 8.0) and lysed by sonication (30-s sonication with an MSE Soniprep at full power, done six times). The protein extract was solubilized and separated by SDS-PAGE, and the gels were stained with Coomassie blue R250 or transferred to nitrocellulose and probed with RhiA antiserum as described previously (3, 7).

**Assay of AHLs.** Cultures were grown for 48 h in TY medium to an OD<sub>600</sub> of 1.0. The cells were removed by centrifugation, and the AHLs were extracted from culture supernatants as described previously (38). AHLs were analyzed by thin-layer chromatography (TLC) as described by Shaw et al. (31) but with use

of *Chromobacterium violaceum* CV026 as the AHL indicator organism (22). CV026 can be used as a biosensor for exogenous AHLs because it produces the purple pigment violacein in response to added AHLs. Culture supernatants and synthetic AHL standards (as 1-mg ml<sup>-1</sup> solutions in acetonitrile) were spotted (2 to 10  $\mu$ l) onto aluminum-backed RP18 reverse-phase TLC plates (Merck) and dried in a stream of air. Samples were separated with 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, dried in air, and overlaid with a thin film of Luria-Bertani soft agar (0.7%, wt/vol) seeded with *C. violaceum* CV026. After overnight incubation at 30°C, AHLs were located by detection of purple spots against a white background.

**Isolation, purification, and chemical characterization of AHLs.** Spent supernatant (4 liters) from stationary-phase cultures was extracted with dichloromethane (supernatant/dichloromethane ratio, 7:3). Dichloromethane was removed by rotary evaporation, and the residue was redissolved in 1.0 ml of acetonitrile and applied to a C<sub>8</sub> reverse-phase semipreparative high-performance liquid chromatography (HPLC) column (Kromasil KR100-5C8 [250 by 8 mm] column; Hichrom, Reading, United Kingdom). Fractions were eluted with a linear gradient of acetonitrile in water (20 to 95%, vol/vol) over a 30-min period at a flow rate of 2 ml/min and monitored at 210 nm. Six fractions (F1 to F6), covering 5-min intervals, were collected and assayed for activity by using a variety of AHL reporter assays, including the *C. violaceum* CV026 detection system (22, 24) and the *E. coli*(pSB401) *luxR* plus *luxI*::*luxCDABE*, and *E. coli*(pSB1075) *lasR* plus *lasI*::*luxCDABE* luminescence detection systems (34, 39). No peaks of activity other than those corresponding with the four spots visualized with the TLC CV026 overlay assay described above were identified. Active fractions were rechromatographed by using an appropriate isocratic mobile phase of acetonitrile in water, and the fractions were assayed for AHL activity. Active subfractions were also reanalyzed on an analytical HPLC apparatus attached to a photodiode array system by using the same isocratic mobile phase (Waters 996 PDA system operating with a Millennium 2010 Chromatography manager; Watford, Hertfordshire, United Kingdom), and both retention time and spectral profiles were compared with those of a series of synthetic AHL standards. Following preparative HPLC, the final active subfractions were analyzed by HPLC-mass spectrometry (HPLC-MS) (Micromass Instruments, Manchester, United Kingdom) using an appropriate isocratic mobile phase. This technique couples the resolving power of C<sub>8</sub> reverse-phase HPLC directly with MS such that the mass of the molecular ion (M + H) and its major component fragments can be determined for a compound with a given retention time. Samples eluting from the HPLC column were ionized by positive-ion atmospheric-pressure chemical-ionization MS and were analyzed at two different cone voltages (18 and 28 eV). The spectra obtained were compared with those of the synthetic AHL standard subjected to the same HPLC-MS conditions.

## RESULTS

**Identification of a novel locus producing AHLs.** Plasmid pIJ1089 carries about 30 kb of DNA from the symbiotic plasmid pRL1J1. Identified genes on pIJ1089 include those involved with nitrogen fixation (*nifHD*) and nodulation (*nodO*, *nodMNT*, *nodFEL*, *nodD*, and *nodABCII*) and the rhizosphere-expressed genes *rhiABC* and *rhiR*. This plasmid directs the production of high levels of the RhiA protein with an  $M_r$  of 24,000 in strain 8401, which lacks a symbiotic plasmid (Fig. 1). pIJ1089 was mutagenized with Tn3HoHo1 or Tn5, the mutated derivatives were conjugated into strain 8401, and protein extracts of the transconjugants were analyzed by SDS-PAGE. Several mutants affected in production of the RhiA protein were identified, and DNA mapping or sequencing from the ends of the transposon confirmed that most of the mutations were in the structural and regulatory genes, *rhiA* and *rhiR*, respectively. However, with two of the mutated derivatives, pIJ1696 and pIJ7790 (carrying Tn3HoHo1 and Tn5, respectively), the sites of transposon insertions mapped to a new locus about 2 kb upstream of *rhiA*. As shown in Fig. 1A, strain 8401/pIJ1089 produces a prominent 24-kDa protein that is absent if *rhiA* is mutated and is very greatly reduced in intensity when *rhiR* is mutated (Fig. 1A, lanes 1, 2, and 4). With 8401/pIJ7790, the level of RhiA protein is significantly decreased (Fig. 1A, lane 3). Immunostaining performed by using antiserum to RhiA (Fig. 1B) confirmed that mutation of the new locus in pIJ7790 significantly reduces the level of *rhiA* expression. Similar observations (data not shown) were made with pIJ1696 (carrying Tn3HoHo1 in the region about 2 kb up-

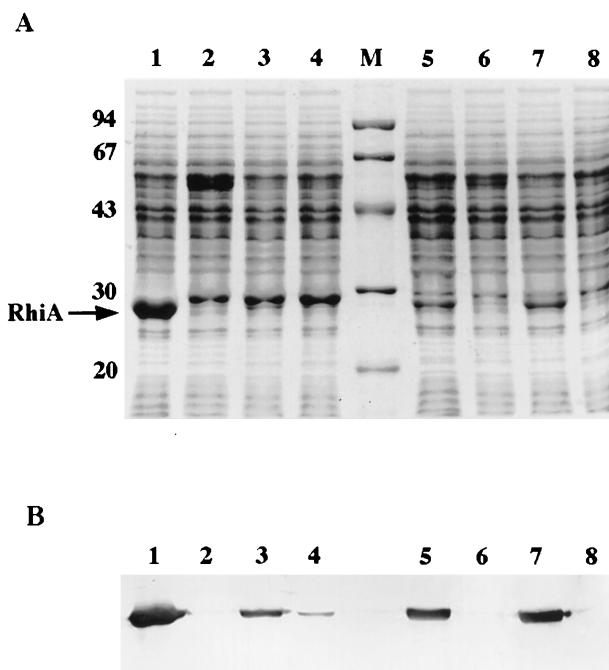


FIG. 1. Effects on RhiA production of mutating *rhiI*. Cell extracts were separated by SDS-PAGE and either stained with Coomassie blue R250 (A) or transferred to nitrocellulose and immunostained with RhiA antiserum followed by a secondary antibody (anti-rabbit immunoglobulin G) coupled to alkaline phosphatase (B). The order of lanes in panel A is the same as that in panel B; 20  $\mu$ g (panel A) or 10  $\mu$ g (panel B) of protein was loaded in each lane. Extracts were from the following: (lane 1) A31/pIJ1089 (wild type); (lane 2) A31/pIJ1243 (*rhiA4*::Tn5); (lane 3) A31/pIJ7790 (*rhiI5*::Tn5); (lane 4) A31/pIJ1242 (*rhiR1*::Tn5); (lane 5) A34 (wild type); (lane 6) A161 (*rhiA4*::Tn5); (lane 7) A721 (*rhiI5*::Tn5); and (lane 8) A160 (*rhiR1*::Tn5). The markers (lane M) have  $M_r$  of 94,000, 67,000, 45,000, 30,000, and 20,000, as indicated.

stream of *rhiA*). We considered that the new locus, mutated in pIJ1696 and pIJ7790, may be involved in production of an AHL that influences expression of *rhiA*.

*A. tumefaciens* C58.00 makes no AHLs detectable by *C. violaceum* CV026 (Fig. 2) and so is a useful strain in testing for AHL production mediated by pIJ1089 and its derivatives (*E. coli* is not a good host for expression of *R. leguminosarum* genes). The culture supernatant of C58.00 carrying pIJ1089 was extracted with dichloromethane and separated by TLC. The chromatogram was overlaid with agar inoculated with the AHL-sensor strain *C. violaceum* CV026 to detect AHLs. As shown here (Fig. 2, lane b), introducing pIJ1089 into C58.00 results in the production of four components that activate pigment production by CV026. It should be noted that the intensity of staining does not reflect the relative amounts of individual AHLs made, since the sensor strain has different sensitivities for different AHLs (21). Two of the spots determined by pIJ1089 comigrate with the synthetic standards C<sub>6</sub>-HSL and C<sub>8</sub>-HSL. The crude extract was fractionated by HPLC by using a linear gradient of acetonitrile in water. When tested by the TLC CV026 overlay assay, fractions 3 and 4 were found to activate violacein production by CV026. Fractions 3 and 4 were further fractionated by using an isocratic acetonitrile-in-water mobile phase (percentages of acetonitrile in water, 25 and 35% [vol/vol], respectively). When active subfractions of fraction 3 were reanalyzed by analytical HPLC, the active compound was found to elute with the same retention time (17 min) and photodiode array absorption spectrum as synthetic C<sub>6</sub>-HSL (data not shown). To confirm the identities

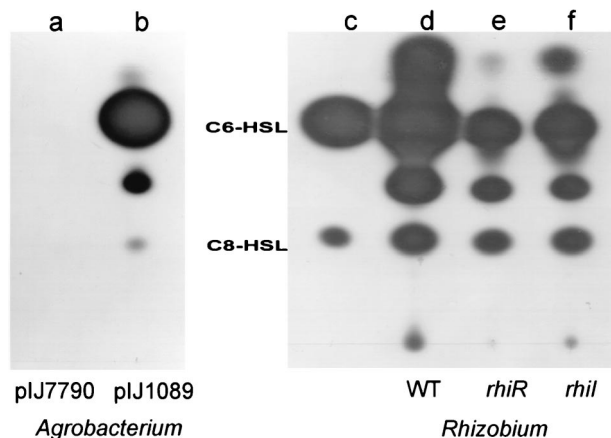


FIG. 2. Identification of AHLs produced by RhlI. AHLs extracted from spent medium were separated by TLC and detected by using an overlay of agar containing *C. violaceum* CV026. Lanes a and b contain extracts of spent culture supernatants from *A. tumefaciens* C58.00 carrying either pIJ7790 (carrying the *rhlI5::Tn5* mutation) (lane a) or pIJ1089 (lane b). Results similar to those seen in lane a (no AHLs were detected) were found with C58.00 carrying no introduced plasmid. Lane c contains the synthetic standards C<sub>6</sub>-HSL and C<sub>8</sub>-HSL. Lanes d, e, and f contain extracts of spent culture supernatants from the *R. leguminosarum* bv. *viciae* strains A34 (wild type), A160 (*rhlR1::Tn5*), and A721 (*rhlI5::Tn5*), respectively.

of the AHLs, active fractions were subjected to HPLC-MS on an isocratic mobile phase (percentages of acetonitrile in water were 25 and 50% [vol/vol] for subfractions from fractions 3 and 4, respectively). Positive-ion atmospheric-pressure chemical-ionization MS for fraction 3 revealed the presence of a molecular ion [M + H] of 200, corresponding to the C<sub>6</sub>-HSL, together with the characteristic fragmentation products at 102 and 99, which correspond to the homoserine lactone moiety and the C<sub>6</sub> acyl side chain [CH<sub>3</sub>(CH<sub>2</sub>)<sub>4</sub>C≡O<sup>+</sup>], respectively. Similar analysis of the active subfraction of fraction 4 confirmed the presence of a molecular ion [M + H] of 228, corresponding to the C<sub>8</sub>-HSL, and breakdown products at 102 and 127, which correspond to the homoserine lactone moiety and the C<sub>8</sub> acyl side chain [CH<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>C≡O<sup>+</sup>], respectively. The two additional putative AHLs detected by the TLC CV026 overlay assay (Fig. 2) did not migrate with any of the known AHLs. Characterization of these compounds is under way.

The plasmid (pIJ7790) defective for RhlA production (Fig. 1) was also defective in the production of all four spots detected by *C. violaceum* CV026 following TLC (Fig. 2, lane a). A similar observation was made with pIJ1696 (data not shown). These results demonstrate that pIJ1089 carries a locus that directs the production of C<sub>6</sub>-HSL and C<sub>8</sub>-HSL (and possibly other AHLs) and that transposon insertions in a region upstream of *rhlA* abolish this phenotype.

**Characterization of *rhlI*.** A 2.5-kb fragment of DNA corresponding to the region mutated by Tn5 and Tn3HoHo1 was subcloned to make pIJ7794, which was confirmed to be involved in AHL production by using *C. violaceum* CV026 as an AHL sensor (data not shown). The DNA sequence of the region was determined, and the sites of the transposon insertions were determined by restriction enzyme mapping and DNA sequencing. Both insertions are located in a short open reading frame (Fig. 3), which encodes a 185-amino-acid protein with similarity to several other proteins (including LuxI) that are involved in AHL production. We called the gene *rhlI* since it is involved in AHL production and mutations of this gene in pIJ1089 decrease *rhlA* expression as judged by the

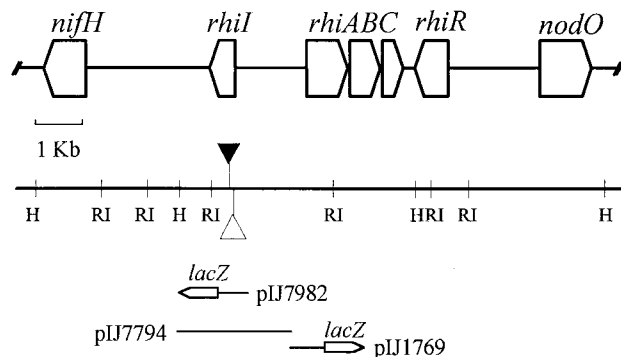


FIG. 3. Map of the *rhi* gene region. The open reading frames corresponding to the *rhlI*, *rhlA*, *rhlB*, *rhlC*, and *rhlR* genes are shown as arrows, and the locations of the *rhlI7::Tn3HoHo1* and *rhlI5::Tn5* insertions are indicated as open and closed triangles, respectively. The DNA cloned to form *rhlI* plasmid pIJ7794 and the *rhlI-lacZ* (pIJ7982) and *rhlA-lacZ* (pIJ1769) fusions is shown.

levels of RhlA protein detected by SDS-PAGE (Fig. 1; compare lanes 1 and 3).

Figure 4 shows an alignment of the predicted sequence of RhlI with the most closely related proteins identified in database searches. Although RhlI shows clear similarities with the LuxI family of proteins, it is evident that RhlI has only about 15 to 20% identity with LuxI (*Vibrio fischeri*), LasI (*Pseudomonas aeruginosa*), Rail (*R. etli*), RhlI (VsmI) (*P. aeruginosa*), and PhzI (*Pseudomonas aureofaciens*). Several other homologs were identified, but these had fewer similarities than those whose alignments are shown in Fig. 4. RhlI contains the highly conserved residues (12, 16, 25) found in multiple LuxI homologs (Arg-24, Phe-27, Trp-33, Glu-43, Asp-45, Asp-48, Arg-68, Glu-99, and Arg-102; numbered with reference to RhlI and marked in Fig. 4). In other RhlI homologs a Phe residue is normally found at position 82 (12), but in RhlI the conservatively substituted residue Tyr is found (Fig. 4).

***rhlI* is regulated by RhlR in a cell density-dependent manner.** A *rhlI-lacZ* reporter plasmid (pIJ7982) was made by cloning a 0.7-kb fragment (containing 0.3 kb of DNA upstream of the predicted *rhlI* translation start site) into the *lacZ* fusion vector pMP220. The expression level of  $\beta$ -galactosidase was measured throughout growth in strain A34, in a derivative of it mutated in *rhlR* (A160), or in a strain lacking the symbiotic plasmid pRL1JI (8401). Parallel experiments were done with the *rhlA-lacZ* plasmid pIJ1769. In A34 there are very low levels of *rhlI* and *rhlA* expression early in growth, but the levels increase markedly in the late exponential phase and reach a plateau in stationary phase (Fig. 5). The expression of *rhlI* is *rhlR* dependent since very low levels of activity are seen with A160 (*rhlR1::Tn5*) or 8401 (which lacks pSym carrying *rhlR*). Therefore, *rhlI* (like *rhlA*) is regulated by RhlR in a cell density-dependent manner. We could identify no sequences upstream of *rhlI* or *rhlA* that showed strong similarity to the 20-bp region of dyad symmetry (*lux* box) found upstream of several genes regulated by LuxR-type proteins (13, 33). However, 45 bp upstream of the proposed translation start site of *rhlI* is a 22-bp region of dyad symmetry (Fig. 6). A similar sequence was found upstream of *rhlA* (Fig. 6), although the center of symmetry was slightly different. It remains to be demonstrated if these regions are involved in the binding of RhlR.

***rhlI* and *rhlA* are induced by C<sub>6</sub>-HSL and C<sub>8</sub>-HSL.** The results presented above indicate that *rhlI* and *rhlA* are likely to be induced by AHLs. To analyze those AHLs that induce *rhlI* and *rhlA*, we made use of derivatives of pIJ1089 carrying *lacZ*

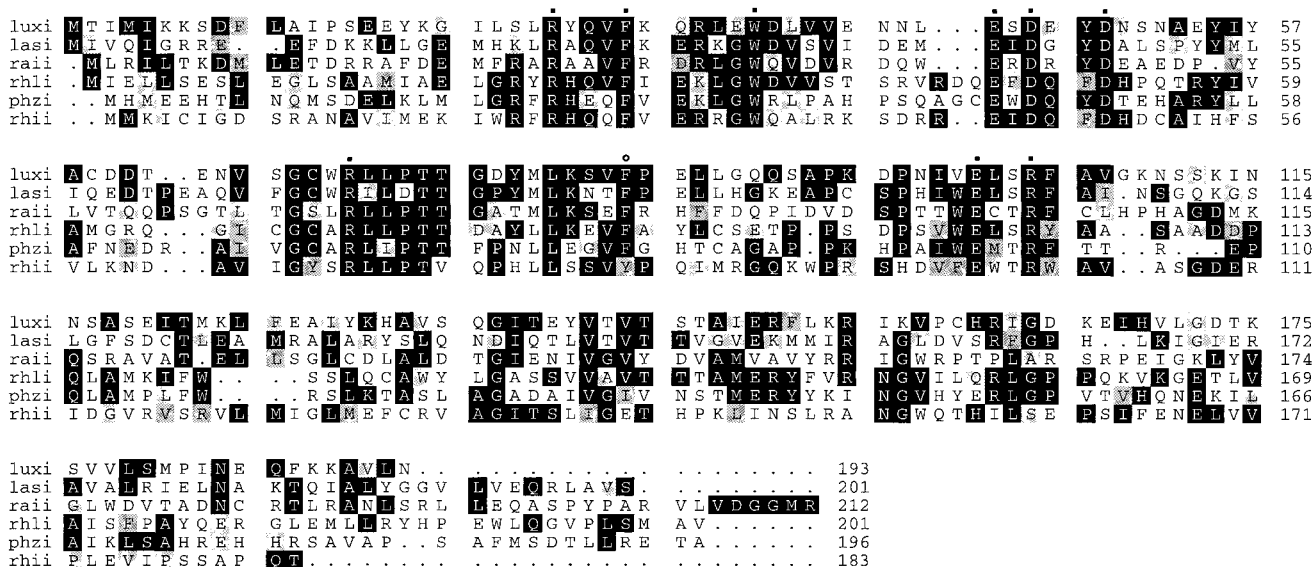


FIG. 4. Alignment of RhiI with related proteins. The predicted protein sequence of RhiI (rhiI) was aligned with the sequences of related proteins by using the Genetics Computer Group programs Pileup and Prettybox. The aligned sequences (and their SwissProt database accession numbers) are LuxI (luxI) (P12747), LasI (lasi) (P33883), RaiI (raii) (U92712), RhlI (rhli) (P54291), and PhzI (phzi) (Q51522). The dots mark residues conserved in multiple LuxI homologs, and the open circle marks a Tyr residue that is usually found to be Phe in other homologs.

transposon insertions. Mapping of Tn3HoHo1 in pIJ1696 revealed that the *lacZ* gene of Tn3HoHo1 was in the same orientation as *rhiI* and under the control of the *rhiI* promoter. Plasmid pIJ1642, a derivative of pIJ1089 carrying *lacZ* under the control of the *rhiA* promoter (*rhiA5*::Tn3HoHo1), was described previously (10). pIJ1696 and pIJ1642 were transferred into the AHL-nonproducer *A. tumefaciens* C58.00. This approach has the advantage of introducing the *rhiR* regulator gene on the same plasmid. (In preliminary experiments we found that there was no production of AHLs by *E. coli* DH5 $\alpha$  carrying pIJ1089 and thus concluded that *E. coli* was not a good host for analysis of *rhiI* expression.) Derivatives of C58.00, carrying pIJ1696 or pIJ1642, were grown to early stationary phase, and the levels of *rhiI* and *rhiA* expression were determined by measuring the levels of  $\beta$ -galactosidase activity in cells. As shown in Table 2, in the absence of added AHLs there was a relatively low level of expression of *rhiI-lacZ*. Addition of C<sub>6</sub>-HSL gave the strongest induction of those AHLs tested, although induction was observed for several other AHLs (Table 2). However, no significant increase in activity was seen with 3OH,C<sub>14:1</sub>-HSL. Similar observations were made with C58.00 carrying pIJ1642 (*rhiA5*::Tn3HoHo1), in that C<sub>6</sub>-HSL was the strongest inducer of *rhiA*, lower levels of induction with C<sub>8</sub>-HSL, 3OH,C<sub>6</sub>-HSL, and 3OH,C<sub>8</sub>-HSL were seen, and no induction with 3OH,C<sub>14:1</sub>-HSL was observed.

These data demonstrate that two of the AHLs produced by RhiI (C<sub>6</sub>-HSL and C<sub>8</sub>-HSL) act as inducers for *rhiI* and *rhiA* expression. However, no induction was observed for 3OH,C<sub>14:1</sub>-HSL, which was previously (15) shown to induce *rhiA-lacZ* expression. This suggests that the previously observed induction by 3OH,C<sub>14:1</sub>-HSL might be an indirect effect caused by regulation of other genes that influence *rhiA* and *rhiI* expression. It should be noted that the levels of expression of *rhiA-lacZ* or *rhiI-lacZ* in *Agrobacterium* strain C58.00 (Table 2) are considerably lower than those seen in *R. leguminosarum* bv. viciae A34 (Fig. 5). This is consistent with the hypothesis that other AHLs made by strain A34 but not made by C58.00 (Fig.

2) contribute to the enhanced expression of *rhiI* and *rhiA* in the *Rhizobium* background.

**Genes on pRL1JI compensate for the absence of *rhiI*.** To analyze the phenotype of an *rhiI* mutant strain, the *rhiI* mutation on pIJ7790 was recombined onto pRL1JI and strain A721 (a derivative of A34 carrying *rhiI5*::Tn5) was constructed. The level of RhiA protein made by stationary-phase cells of A721 was analyzed: whereas mutation of *rhiI* on pIJ1089 significantly reduced the levels of RhiA formation (Fig. 1, lane 3), mutation of *rhiI* on pRL1JI had little or no observed effect on RhiA formation (Fig. 1, lane 7). The high level of RhiA in A721 (*rhiI* mutation on pRL1JI) compared with the level in strain 8401/pIJ7790 (*rhiI* mutation on pIJ1089) indicates that there may be a locus on pRL1JI that compensates for the absence of *rhiI* and that this locus is not contained in the 30-kb region of pRL1JI cloned in pIJ1089.

Measurements of the expression of *rhiA-lacZ* (pIJ1769) or *rhiI-lacZ* (pIJ7790) in A721 confirmed that the *rhiI* mutation in A721 does not reduce *rhiA* or *rhiI* expression (Fig. 5) compared with that for the control strain (A34). Therefore, although those AHLs produced by RhiI can induce *rhiI* and *rhiA* gene expression in strain C58.00 (Table 2), mutation of *rhiI* has little effect on *rhiI* and *rhiA* expression in the A34 background. These observations are consistent with a model in which *rhiA* and *rhiI* are regulated by RhiR not only in response to RhiI-made AHLs (such as C<sub>6</sub>-HSL and C<sub>8</sub>-HSL) but also in response to other AHLs that could be made by a product of another gene located elsewhere in the genome of A34. We used *C. violaceum* CV026 to analyze AHLs made by strain A34 and the derivatives of it carrying mutations in *rhiI* (A721) or *rhiR* (A160). As shown (Fig. 2, lane d), strain A34 makes many different compounds that are detected by this system; we estimate that (in addition to 3OH,C<sub>14:1</sub>-HSL that is not detected) there are at least six components that activate pigment production by *C. violaceum* CV026. Other components might be present but are not detected by this reporter system (22). Mutation of *rhiI* (Fig. 2, lane f) reduces the amount of C<sub>6</sub>-HSL,

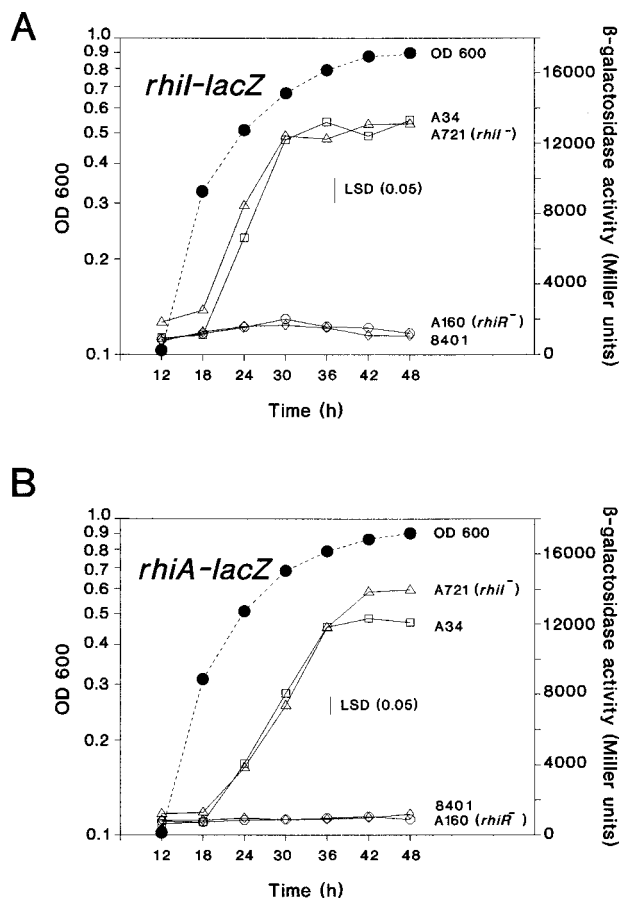


FIG. 5. Expression of *rhl-lacZ* and *rhlA-lacZ*. The expression of *rhl-lacZ* (pIJ7794) (A) or *rhlA-lacZ* (pIJ1769) (B) was analyzed by measuring  $\beta$ -galactosidase throughout growth of strains A34 (wild type), 8401 (strain A34 lacking pRL1JI), A160 (*rhlR*::Tn5), and A721 (*rhlI5*::Tn5). The growth curves (OD<sub>600</sub>) shown as broken lines correspond to those obtained with A34/pIJ7794 or A34/pIJ1769; the growth of the other strains was very similar. LSD, least significant difference.

but an active component with the same mobility as C<sub>6</sub>-HSL is clearly made by the *rhlI* mutant (A721). This is consistent with the observations on RhlA production and *rhl-lacZ* or *rhlA-lacZ* expression, which indicate that another locus in A721 may be involved in formation of C<sub>6</sub>-HSL. Mutation of *rhlR* (A160) has a slightly stronger effect on AHL production (Fig. 2, lane e) than mutation of *rhlI*. The difference between the *rhlR* and *rhlI* mutants could be explained if RhlR influences the expression of a gene present at another locus and involved in AHL production.

*C. violaceum* CV026 does not detect 3OH,C<sub>14:1</sub>-HSL, and so we measured the effect of mutating *rhlI* on production of this AHL using A34 as a sensor strain in a bacteriocin-like assay (35). Strain A721 did not induce a zone of growth inhibition, indicating that repression of 3OH,C<sub>14:1</sub>-HSL occurred normally and therefore mutation of *rhlI* did not affect the ability of pRL1JI to repress production of this AHL (data not shown). Strain A721 was also used as a sensor (lawn) in a similar assay, and its growth was as sensitive as that of the control strain (A34) to growth inhibition by 3OH,C<sub>14:1</sub>-HSL produced by strain 8401, showing that mutation of *rhlI* does not affect the growth sensitivity of A34 to 3OH,C<sub>14:1</sub>-HSL.

**Influence of *rhlI* on nodulation.** Previous work with *rhlA-lacZ* fusions demonstrated that flavonoid inducers of *nod* gene



FIG. 6. Conserved DNA sequences found upstream of *rhlI* and *rhlA*. The sequences shown are found 45 and 146 bp upstream of the predicted translation start sites of *rhlI* and *rhlA*, respectively. Conserved residues (·) are marked, and the dyad symmetry is indicated with arrows.

expression decreased *rhlA* expression by about 50% and that this decrease was *nodD* dependent (5, 10). We measured expression of *rhl-lacZ* using pIJ7982 in the presence and absence of the *nod* gene inducer hesperetin, under conditions similar to those shown in Fig. 5A. After 42 h of growth, the level of  $\beta$ -galactosidase activity in the cells grown with hesperetin (6,800  $\pm$  610 U) was about half that seen when hesperetin was not added (11,300  $\pm$  840 U). Therefore, like that of *rhlA*, *rhlI* expression is decreased by inducers of *nod* gene expression. No significant effect of hesperetin on expression of the *rhl-lacZ* fusion in the *nodD* mutant A57 was observed (data not shown), confirming that the hesperetin-induced reduction of *rhl-lacZ* expression is *nodD* dependent.

We tested nodulation ability of the *rhlI* mutant (A721) relative to that of the control strain A34. The mutant formed normal nitrogen-fixing nodules on peas; the final number of nodules formed was slightly (but significantly) higher than that for the control, although the rate of nodule formation was similar to that for the control (Fig. 7).

## DISCUSSION

The *rhlABC* operon is conserved in all *R. leguminosarum* bv. *viciae* strains tested and has not been found in other rhizobia. This, taken together with the location of the *rhl* gene cluster (between the *nod* and *nif* genes), indicates that these genes may play some kind of role in the interaction between *R. leguminosarum* bv. *viciae* and at least some of its host legumes.

It is now evident that the *rhlABC* genes are regulated by RhlR in response to AHLs made by RhlI. The *rhlI* gene is regulated in the same way, thereby forming a positive autoregulatory loop that results in high levels of expression from the *rhlI* and *rhlA-lacZ* promoters. This explains the very high levels of RhlA protein detected in late-stationary-phase cells, in which it is certainly one of the most abundant proteins (7). However, *rhlA* expression is not totally dependent on *rhlI* since

TABLE 2. Effects of added AHLs on *rhl-lacZ* and *rhlA-lacZ* expression<sup>a</sup>

AHL tested	Expression (Miller units) of:	
	<i>rhl-lacZ</i>	<i>rhlA-lacZ</i>
None (negative control)	369.78	198.98
C <sub>6</sub> -HSL	2,493.28 <sup>b</sup>	1,079.54 <sup>b</sup>
C <sub>8</sub> -HSL	1,585.57 <sup>b</sup>	546.43 <sup>b</sup>
3O,C <sub>6</sub> -HSL	896.01 <sup>b</sup>	196.35
3O,C <sub>8</sub> -HSL	1,191.00 <sup>b</sup>	332.16 <sup>b</sup>
3OH,C <sub>4</sub> -HSL	544.89 <sup>b</sup>	154.39
3OH,C <sub>14</sub> -HSL	484.12	151.94
LSD ( <i>P</i> < 0.05)	170.66	82.45

<sup>a</sup> AHLs were tested in *Agrobacterium* sp. strain C58.00 carrying pIJ1642 or pIJ1696, using a final concentration of 0.1  $\mu$ M each AHL.

<sup>b</sup> Values are significantly different from that for the negative control (Student's *t* test, *P* < 0.05).

<sup>c</sup> LSD, least significant difference.

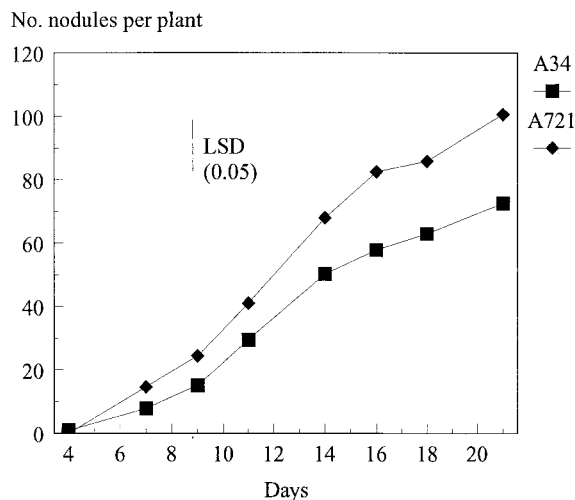


FIG. 7. Effect of an *rhiI* mutation on nodulation. The average numbers of nodules formed by strains A34 (wild type) and A721 (*rhiI15::Tn5*) on Frisson peas are shown. The data shown are averages from one data set obtained with 16 plants. Similar results were found with two separate data sets. The difference in the final numbers of nodules formed is statistically significant ( $P < 0.05$ ). LSD, least significant difference.

there appears to be another AHL production locus which can form AHLs that stimulate the expression of both the *rhiABC* operon and *rhiI*. Since *rhiR* mutants show very little expression of *rhiI* or *rhiABC*, it is evident that although there are other loci for AHL production, any regulatory genes that might be associated with those loci are not able to induce *rhiABC* or *rhiI* expression. However, there could be an indirect effect on *rhiABC* expression since AHLs, made by products of genes other than *rhiI*, can stimulate RhiR to induce *rhiI* and *rhiABC* expression. Thus, there is likely to be a degree of cross talk between different AHL production loci.

Previously the *rhiA* promoter was observed to be induced when 3OH,C<sub>14:1</sub>-HSL was added to wild-type cells during early-exponential-phase growth (15). However, RhiR activates *rhiA* in response to C<sub>6</sub>-HSL and C<sub>8</sub>-HSL but not 3OH,C<sub>14:1</sub>-HSL. Therefore, the most-probable explanation for the previous results is that 3OH,C<sub>14:1</sub>-HSL induces the expression of other AHLs, which in turn activate RhiR-mediated *rhiABC* and *rhiI* expression. The fact that *rhiI* and *rhiR* mutants still produce many short-chain AHLs is good evidence that there is at least one other AHL production locus in *R. leguminosarum* bv. viciae; indeed, in other (unpublished) work, we have cloned four AHL production loci from strain A34. Two other *luxI*-like genes in rhizobia have been described. In *R. etli* the *rail* gene was sequenced and shown to be involved in the formation of several (chemically uncharacterized) AHLs (26). In *Rhizobium* sp. strain NGR234, a *traI* gene was identified in a symbiotic plasmid genome-sequencing project (11). Although RhiI described here is in the same family as these two proteins, it is not much more similar to RaiI or TraI proteins from rhizobia than to related proteins from several other bacteria (Fig. 4). *R. leguminosarum* bv. viciae strain A34 may have, in addition to *rhiI*, other AHL production genes homologous to *traI* and/or *rail*. Perhaps the diversity of AHL production systems in *Rhizobium* may be related to the fact that many strains harbor multiple large plasmids. Different plasmids may have different AHL production systems, and strain 8401 (lacking a symbiosis plasmid) contains two plasmids thought to be greater than 300

and 500 kb in size (20). It remains to be determined if these plasmids harbor genes involved in AHL production.

It is not clear why *R. leguminosarum* bv. viciae should have the *rhiI-rhiR* regulatory system to induce expression of the *rhiABC* operon, although several lines of evidence relate this to some aspect of the interaction with leguminous plants. The observation that flavonoids which induce *nod* gene expression reduce *rhiI* expression suggests that the plant has the potential to influence the level of AHL production. However, the effect of flavonoids on *rhi* gene expression depends on the *R. leguminosarum* bv. viciae *nod* gene regulator NodD (10), indicating that the bacteria influence this decrease in *rhiI* and *rhiABC* expression. This is somewhat different from the observed inhibition of quorum-sensing regulated genes by halogenated furanones, which are thought to act as competitive inhibitors of AHL binding to LuxR-type regulators (14).

We do not yet know the biochemical role of the *rhiABC* gene products or why they should be regulated in a cell density-dependent manner. It is evident that in some way they influence the interaction with the plant since mutation of *rhiA* or *rhiR* significantly reduced nodulation in a strain lacking the *nodFEL* genes (5). Paradoxically, mutation of *rhiI* increased the final number of nodules formed. In *R. etli*, mutation of the *rail* gene, which is also involved in AHL production, resulted in increased levels of bean nodulation (26). Thus, for two separate *Rhizobium*-legume interactions there is independent evidence that production of (at least) some of the AHLs inhibits nodulation under the growth conditions tested.

In the absence of significant protein sequence similarities to any other proteins of known function, it is difficult to predict the role of RhiA, RhiB, and RhiC. The RhiC protein appears to be located in the periplasm, possibly suggesting a role for the uptake of some metabolite, but our tests of growth of *rhiABC* mutants on various carbon sources have not identified any clear differences from the growth of the isogenic control strain (9a). It is evident that in *R. leguminosarum* bv. viciae quorum-sensing-based regulation is complex and may share similarities with the cascade of quorum-sensing-regulated genes in *P. aeruginosa* and *V. fischeri*. The reason for such complexity of regulation and for the apparent degree of redundancy of AHL production is not clear, and its elucidation will require characterization of the other AHL production loci in *R. leguminosarum* bv. viciae.

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