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Quorum Sensing in *Rhizobium* sp. Strain NGR234 Regulates Conjugal Transfer (*tra*) Gene Expression and Influences Growth Rate

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***Rhizobium* sp. strain NGR234 forms symbiotic, nitrogen-fixing nodules on a wide range of legumes via functions largely encoded by the plasmid pNGR234a. The pNGR234a sequence revealed a region encoding plasmid replication (*rep*) and conjugal transfer (*tra*) functions similar to those encoded by the *rep* and *tra* genes from the tumor-inducing (Ti) plasmids of *Agrobacterium tumefaciens*, including homologues of the Ti plasmid quorum-sensing regulators TraI, TraR, and TraM. In *A. tumefaciens*, TraI, a LuxI-type protein, catalyzes synthesis of the acylated homoserine lactone (acyl-HSL) *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL). TraR binds 3-oxo-C8-HSL and activates expression of Ti plasmid *tra* and *rep* genes, increasing conjugation and copy number at high population densities. TraM prevents this activation under noninducing conditions. Although the pNGR234a TraR, TraI, and TraM appear to function similarly to their *A. tumefaciens* counterparts, the TraR and TraM orthologues are not cross-functional, and the quorum-sensing systems have differences. NGR234 TraI synthesizes an acyl-HSL likely to be 3-oxo-C8-HSL, but *traI* mutants and a pNGR234a-cured derivative produce low levels of a similar acyl-HSL and another, more hydrophobic signal molecule. TraR activates expression of several pNGR234a *tra* operons in response to 3-oxo-C8-HSL and is inhibited by TraM. However, one of the pNGR234a *tra* operons is not activated by TraR, and conjugal efficiency is not affected by TraR and 3-oxo-C8-HSL. The growth rate of NGR234 is significantly decreased by TraR and 3-oxo-C8-HSL through functions encoded elsewhere in the NGR234 genome.**

Quorum sensing is a regulatory process by which bacteria monitor their population density via the production and subsequent perception of diffusible signal molecules. Many gram-negative members of the *Proteobacteria* employ acylated homoserine lactones (acyl-HSLs) as quorum-sensing signal molecules (see references 15, 16, and 68 for reviews). Acyl-HSLs are synthesized in the bacterial cytoplasm by enzymes that are often members of the LuxI family (14, 35). The acyl-HSLs can diffuse passively across the cell membrane but in some cases are assisted by membrane efflux systems (9, 32). When the population of signal-producing cells is diffuse, the relative intracellular acyl-HSL concentration remains low. Increased population density leads to elevated acyl-HSL levels, eventually driving interaction with a cytoplasmic receptor protein that is usually a member of the LuxR family of transcriptional regulators (60). In most cases, association with the acyl-HSL stimulates the DNA binding activity of the LuxR protein and transcriptional activation of target genes (66, 75). Although the general quorum-sensing mechanism is well conserved among different members of the *Proteobacteria*, the target functions can vary considerably. These functions are frequently involved in host-microbe interactions (49).

Several different genera of the rhizobia, members of the family *Rhizobiaceae*, cause the formation of symbiotic, nitrogen-fixing nodules on leguminous plants. The complex developmental process of nodule formation involves sequential bacterial-plant signal exchange, requiring specific nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) genes (see references 25, 38, and 50 for extensive reviews). Among the *Rhizobiaceae*, acyl-HSL quorum sensing is quite prevalent. The best studied of these systems in a member of the *Rhizobiaceae* is found in the plant pathogen *Agrobacterium tumefaciens*, which causes crown gall disease (71). The *A. tumefaciens* quorum sensor requires the acyl-HSL synthase TraI, which directs synthesis of *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL). The acyl-HSL-responsive regulator TraR associates with 3-oxo-C8-HSL, binds to DNA sequences called *tra* boxes upstream of regulated promoters, and activates transcription (Fig. 1A) (19, 20, 29, 51, 73). A third regulatory protein, TraM, functions as an antiactivator, preventing TraR from activating target genes under noninducing conditions (13, 28, 62). TraR, TraI, and TraM are encoded on the tumor-inducing (Ti) virulence plasmid and regulate the conjugal transfer (*tra*) and vegetative replication (*rep*) functions of this plasmid (19, 29, 36).

Acyl-HSL quorum sensing in the symbiotic rhizobia is generally more complex than in *A. tumefaciens*. There are often multiple LuxR-type and LuxI-type proteins active within the same strain, organized in a regulatory hierarchy. *Rhizobium leguminosarum* bv. viciae synthesizes four to six different acyl-HSLs via the activity of four LuxI-type proteins and has at least five LuxR-type proteins (6, 37). *Rhizobium etli* CNPAF512 and *Sinorhizobium meliloti* AK631 synthesize seven and nine different acyl-HSLs, respectively, and encode multiple LuxR-LuxI type regulators (2, 7, 42, 56). In *R. leguminosarum* the quorum sensors are arranged in a hierarchal network that

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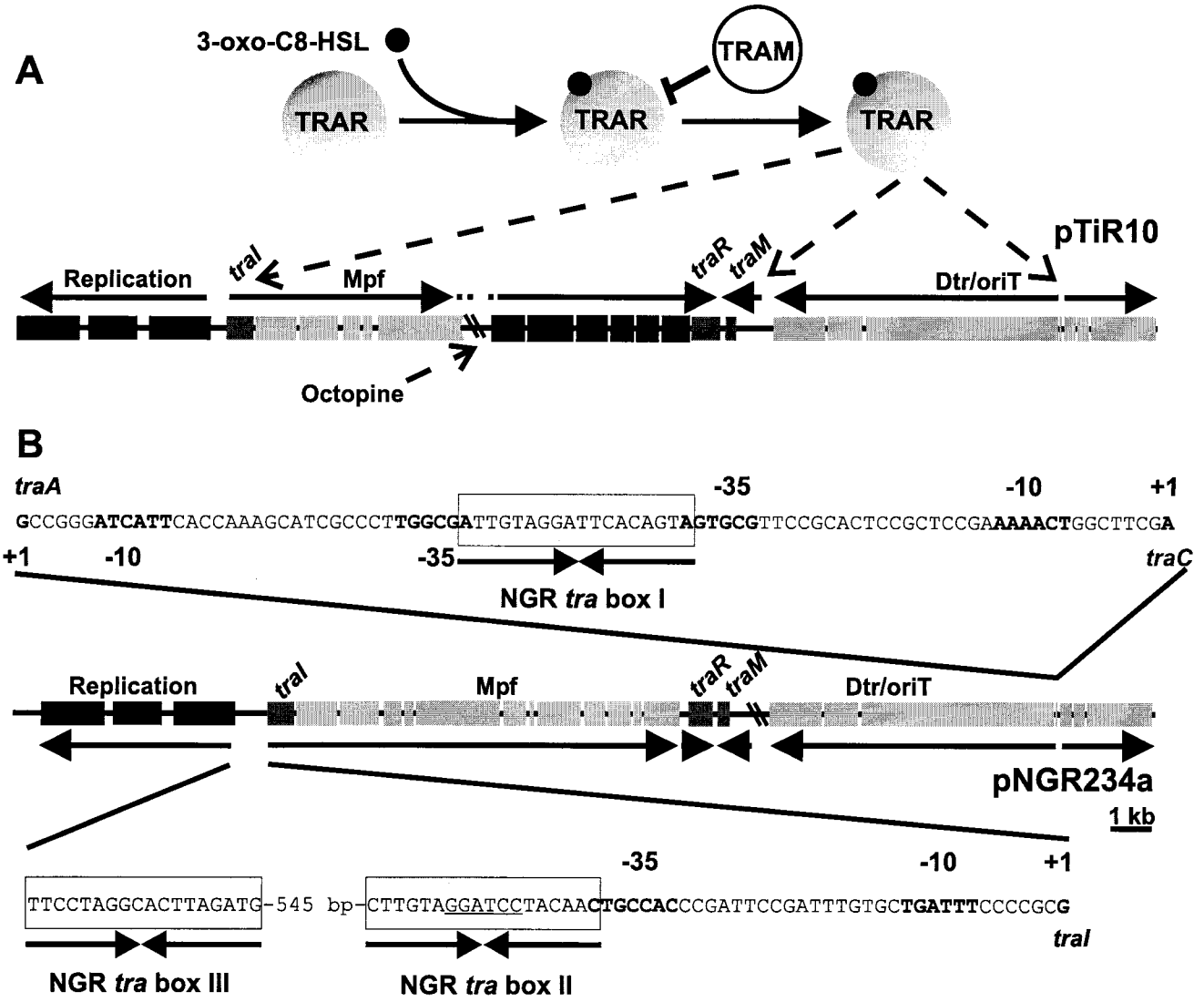

FIG. 1. Conjugal transfer-replication clusters of an *A. tumefaciens* octopine-type Ti plasmid and pNGR234a. Conserved genes are aligned with their counterparts and shaded similarly. Large arrows above lines indicate operons and predicted operons for Ti plasmids and pNGR234a, respectively. Dashed arrows indicate likely regulatory pathways based on Ti plasmid systems. Hash marks along linear maps represent 57 kb for pTiR10 and 6 kb for pNGR234a. (A) Genetic map of pTiR10 *tra-rep* region and model of TraR regulation. (B) Exploded views of presumptive promoter sequences of the Dtr operons initiating with *traA* and *traC* and the Mpf operon initiating with *traI*. Likely promoter consensus elements (−10, −35, and TSP) are indicated in boldface, and presumptive *tra* box elements are boxed with arrows indicating their dyad symmetry. Start sites for operons initiating with *traC* and *traI* were experimentally determined, while the start site for the *traA*-initiated operon is estimated based on proximity to *tra* box I and the analogous location on pTiR10. The *Bam*HI site disrupted in the pBDJ7 construct is underlined in NGR *tra* box II. The sequence designated NGR *tra* box III has been identified by sequence analysis alone. Mpf, mating pair formation; Dtr, DNA transfer and replication; *oriT*, origin of transfer.

coordinates conjugal transfer gene expression on the pRL1JI symbiotic plasmid, rhizosphere-specific gene expression, and other, as-yet-undefined functions (69). Rhizobial mutants deficient in quorum sensing exhibit a variety of defects during the symbiotic nodulation of plants, ranging from modest to severe (7).

The wide-host-range nodulator *Rhizobium* sp. strain NGR234 can nodulate over 120 different legume species and at least one nonlegume (54). The NGR234 genome is comprised of two large episomes, pNGR234a (536,165 bp) and pNGR234b (approximately 2 Mb) and a chromosome (approximately 3.5 Mb), all of which can exist independently or as cointegrates in every possible combination (11, 44). The majority of symbiotic functions are encoded on pNGR234a. The complete pNGR234a sequence revealed the presence of (i) *nod*, *fix*, and *nif* genes that direct the plant interaction; (ii) genes with presumptive functions in the symbiotic process; and (iii) a cluster of genes homologous to Ti plasmid *rep* and *tra* genes of *A. tumefaciens* (12). The pNGR234a *tra-rep* cluster is homologous and syntenous to its counterparts on the Ti plasmids over a discrete region including a pair of divergent operons encoding predicted DNA transfer and replication (Dtr)

TABLE 1. Relevant strains and plasmids

Bacterium or plasmid	Relevant features	Reference or source
Bacteria		
E. coli DH5αF′	Standard cloning host	72
E. coli S17-1/λ*pir*	IncP conjugal donor, Sm^r	31
C. violaceum CV026	Acyl-HSL reporter strain	45
Rhizobium sp. strain NGR234	Wild-type New Guinea isolate	64
Rhizobium sp. strain NGR234-ANU265	pNGR234a cured, Sp^r Sm^r	47
A. tumefaciens NTL4	Ti plasmidless derivative, nopaline chromosomal background	39
A. tumefaciens C58 C1RS	Plasmidless derivative of nopaline-type strain C58, Sm^r Rif^r	8
Rhizobium sp. strain NGR234-BDJ3	NGR234 with integrated pCF429, Km^r	This study
Rhizobium sp. strain NGR234-XH100	NGR234 with integrated pXH100 disrupting *traI,* Km^r	This study
Rhizobium sp. strain NGR234-XH101	NGR234 with integrated pXH101 retaining functional *traI,* Km^r	This study
Plasmids		
pCR-Script	PCR product cloning vector, Ap^r	Stratagene Inc.
pBBR1-MCS5	BHR P_{lac} expression vector, Gm^r	34
pSW172	BHR IncP plasmid, Tc^r	4
pSW213	BHR IncP plasmid, $lacI^q$ Tc^r	4
pVIK112	*lacZ* transcriptional fusion integration vector, Km^r	31
pRA301	BHR *lacZ* translational fusion vector, Sp^r, IncP derivative	1
pCF218	BHR IncP $traR_{Ti}$ expression plasmid, Tc^r	19
pCF372	BHR IncW $traI_{Ti}$-*lacZ* fusion plasmid, Sp^r	17
pGB200	BHR pBBR1-MCS5 derivative carrying P_{lac}-$traM_{Ti}$ fusion, Gm^r	62
pCF422	pCR-Script derivative carrying full-length PCR-amplified $traM_{NGR}$ gene, Ap^r	This study
pCF424	BHR pBBR1-MCS5 derivative, carrying P_{lac}-$traM_{NGR}$ fusion, Gm^r	This study
pCF429	Derivative of pVIK112 carrying full-length $traM_{NGR}$ gene from pCF422, Km^r	This study
pBDJ3	pRA301 derivative, NGR234 P_{traI}-*lacZ,* Sp^r	This study
pBDJ6	pBBR1-MCS5 derivative, P_{lac}-$traR_{NGR}$, Gm^r	This study
pBDJ7	pBDJ3 derivative, tra_{NGR} box (*Bam* HI) fill-in, Sp^r	This study
pDP101	pBDJ6 derivative with P_{lac}-*traR*-*traM,* Gm^r	This study
pXH100	Internal fragment of pNGR234a *traI* fused to *lacZ* on pVIK112, Km^r	This study
pXH101	Truncated fragment of pNGR234a *traI* fused to *lacZ* on pVIK112 (including intact 5′ end), Km^r	This study
pXH102	pBSK with internal *traI* fragment of pNGR234a in *Eco*RI/*Xba*I sites, Ap^r	This study
pXH103	pBSK with *traI* fragment (including intact 5′ end) of pNGR234a in *Eco*RI/*Xba*I sites, Ap^r	This study

functions (including the origin of transfer, *oriT*) and a separate but linked region with a pair of divergent operons encoding predicted *rep* and mating pair formation (Mpf) functions (Fig. 1B). Regulatory genes within this region are also conserved with those on the *A. tumefaciens* Ti plasmids, including the *traR*, *traI*, and *traM* regulatory genes.

The pNGR234a quorum-sensing mechanism was investigated in this study. Although there are interesting differences, the pNGR234a quorum-sensing mechanism is similar to that of *A. tumefaciens* Ti plasmids, and expression of *traR* limits expression of pNGR234a *tra* genes. Additionally, the pNGR234a-encoded quorum sensor significantly influences the growth rate of NGR234, providing a mechanism by which quorum sensing and population growth may be integrated.

MATERIALS AND METHODS

Strains, plasmids, reagents, and growth conditions. All strains and plasmids used in this study are described in Table 1. Buffers, antibiotics, and microbiological media were obtained from Fisher Scientific (Pittsburgh, Pa.) and Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled nucleotides were purchased from Amersham Pharmacia Biotech (Piscataway, N.J.). DNA manipulations were performed by standard protocols (57) with enzymes purchased from New England Biolabs and Epicentre Technologies (Madison, Wis.). Sequenase version 2.0 was obtained from United States Biochemical Corp. (Cleveland, Ohio). Thermostable DNA polymerases, *Taq* and *Pfu*, for PCR were obtained from Panvera (Madison, Wis.) and Stratagene Corp. (La Jolla, Calif.), respectively. Custom oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa), and standard DNA sequencing was performed on an ABI 3700 automated sequencer in the Indiana Molecular Biology Institute (Bloomington, Ind.). Crude 3-oxo-C8-HSL (designated *Agrobacterium* autoinducer [AAI]) was prepared as the cell-free culture filtrate of strain KYC6(pCF218), which strongly overproduces the pheromone (19). Organically synthesized 3-oxo-C8-HSL was generously provided by Anatol Eberhard, and all other acyl-HSLs were purchased from Quorum Sciences Inc. (Iowa City, Iowa).

NGR234 and derivatives were cultivated at 28°C in TY complex medium (5 g of Bacto Tryptone per liter, 3 g of yeast extract per liter, 1.2 g of $CaCl_2 \cdot 2H_2O$ per liter [pH 6.9]) and a modified M9 minimal salts medium (*Rhizobium* minimal medium [RMM]) supplemented with 15 mM glucose and with thiamine, calcium pantothenate, and biotin at 1 mg/ml each (3). *A. tumefaciens* strains were grown at 30°C in either Luria-Bertani medium or AT minimal salts medium with 0.5% glucose and 15 mM $(NH_4)_2SO_4$ (ATGN) (63). Antibiotics were used at the following concentrations (micrograms per milliliter): (i) for *Escherichia coli*, ampicillin (100), gentamicin (25), kanamycin (25), spectinomycin (100), and tetracycline (10); (ii) for *A. tumefaciens*, gentamicin (800), spectinomycin (50), rifampin (50), streptomycin (1,000), and tetracycline (5); and (iii) for NGR234, gentamicin (50), kanamycin (25), rifampin (150), streptomycin (1,000), spectinomycin (25), and tetracycline (10).

Plasmids were electroporated into *A. tumefaciens* by using a standard method (46). Introduction of plasmids into NGR234 was performed by conjugation or by

electroporation with the *A. tumefaciens* protocol modified by washing the cells in 10% glycerol rather than water. Following electroporation, the NGR234 cell suspension was diluted in TY medium and usually incubated for 1 to 2 h at 28°C prior to plating on selective medium.

Isolation and manipulation of NGR234 quorum-sensing genes. The complete coding sequences of *traR* and *traM* and several segments of *traI* from pNGR234a were isolated by PCR with NGR234 genomic DNA and primers designed by using the pNGR234a DNA sequence (12).

To generate a broad-host-range (BHR) derivative expressing the pNGR234a *traR* gene from the P_{lac} promoter on the pBBR1-MCS5 vector, the *traR* coding sequence was amplified from total genomic DNA by PCR with two oligonucleotides, 5′-cgcggtaaccc**tgataa**CGGCAAAAGAT**AGGAG**AGA**ATG**TCC and 5′-gcgtctaga**TCA**GACCAGGCCGCGATCCTTG (for all oligonucleotides, the pNGR234a sequence is in uppercase). The first oligonucleotide incorporates (i) a *Kpn*I site (underlined) upstream of the coding sequence, (ii) a tandem pair of stop codons (in boldface) to prevent readthrough from the *lacZ*α peptide of pBBR1-MCS5, and (iii) the first two *traR* codons plus associated translational signals (in boldface). The second, downstream oligonucleotide includes the native stop codon (in boldface) flanked by an engineered *Xba*I site (underlined). PCR amplification generated a 755-bp product that was purified, digested with *Kpn*I and *Xba*I, and ligated with similarly digested pBBR1-MCS5 to generate pBDJ6. The *traR* coding sequence of the pBDJ6 fusion construct was verified as correct by DNA sequencing.

The *traM* gene from pNGR234a was PCR amplified from genomic DNA by using oligonucleotides 5′-ggcggtaccc**tgataa**acaggaaacagctATGAACGATATGGGCTCA TCCGAGG and 5′-ggcctgcag**CTA**GTTGTCATCGTCGAAAGGC. The first oligonucleotide carries an engineered *Kpn*I site (underlined), followed by two stop codons (in boldface), the Shine-Dalgarno sequence from the *Escherichia coli lacZ* gene (underlined), and the first seven codons of *traM*. The second oligonucleotide includes the native stop codon (boldface) flanked with a *Pst*I site (underlined). The purified 361-bp amplification product was cloned by using the PCR-Script Amp cloning system (Stratagene Corp.), blunt-end ligating the product with linearized pCR-Script vector to generate pCF422. The *traM* insert in pCF422 was sequenced and found to be identical to the published sequence (12). pCF422 was digested with *Kpn*I and *Pst*I and ligated with similarly digested pBBR1-MCS5 to produce pCF424, a BHR derivative with a P_{lac}-*traM* fusion. To generate a single plasmid carrying *traR* and *traM* from pNGR234a, pCF422 was linearized at its single *Eag*I site, the 5′ extensions were filled in by using the Klenow fragment of DNA polymerase I, and the entire plasmid was ligated into the *Sma*I site of pBDJ6 immediately downstream of the *traR* coding sequence. This BHR plasmid, designated pDP101, expresses a P_{lac}-*traR-traM* fusion. Lastly, to generate a recombinational insertion plasmid, the *traM* insert in pCF422 was excised as a *Kpn*I fragment and ligated with *Kpn*I-cleaved pVIK112, an R6K-derived suicide vector that can be maintained only in *E. coli* hosts that express the *trans*-acting R6K replication factor gene *pir* (31), to generate pCF429.

The *traI* promoter region from pNGR234a was isolated by using oligonucleotides 5′-ccggaattcTGAATATATTCGAGTTCTGAGTTGC and 5′-ggctctagaC TG**CAT**GAATT**TCTCC**GTCGTTGTTGG. The first oligonucleotide contains an *Eco*RI site (underlined) and is positioned 495 bp upstream of the predicted *traI* start codon. The second oligonucleotide carries the native translational signals of *traI* (in boldface) flanked by an *Xba*I cleavage site (underlined). The 513-bp amplification product was purified, digested with *Eco*RI and *Xba*I, and ligated with similarly digested pRA301, an IncP BHR plasmid, generating a BHR_3 *PtraI-lacZ* fusion plasmid designated pBDJ3 (1). In order to test the role of the presumptive *tra* box proximal to *traI*, pBDJ3 was cleaved at its single *Bam*HI site located in the exact center of the *tra* box (Fig. 1B), the 5′ overhangs were filled in with the Klenow fragment of DNA polymerase I, and the plasmid was recircularized to generate pBDJ7. Two fragments of the *traI* gene were PCR amplified from NGR234 genomic DNA for recombinational mutagenesis. An internal fragment truncated on both ends of the *traI* coding sequence was isolated by using oligonucleotides 5′-cgctctagaTTTGCCGGCAGAGTGCCCGC and 5′-cgcgaattcGAAGTCAATGTCGTCGGAGG. The first oligonucleotide anneals upstream of the predicted *traI* stop codon and is flanked by an *Xba*I site (underlined). The second oligonucleotide contains an *Eco*RI site (underlined) and anneals 105 bp downstream of the predicted *traI* start codon. The predicted PCR product is 464 bp. A larger fragment with all of the *traI* upstream sequence, including the promoter region, but truncated in the carboxy-terminal coding region was PCR amplified by using the oligonucleotide 5′-ccggaattcTGAATAT ATTCGAGTTCTGAGTTGC and the same second oligonucleotide as used for the doubly truncated product. The first oligonucleotide for this larger product also has an *Eco*RI site (underlined) but is positioned 495 bp upstream of the predicted *traI* start codon. The expected product of this fragment is 1,064 bp. The two amplification products were purified, digested with *Eco*RI and *Xba*I, and ligated with similarly digested pVIK112 integration vector, generating pXH100 and pXH101. These plasmids also create *traI*::*lacZ* transcriptional fusions at the identical position. The same PCR fragments used to generate pXH100 and pXH101 were also used to create the pBSK(+) derivatives pXH102 and pXH103, respectively.

Recombinational integration of plasmids into pNGR234a. To integrate plasmids into pNGR234a, the plasmids carrying segments of pNGR234a were conjugated from the BHR conjugal donor *E. coli* S17-1/λ*pir* into NGR234-ACF1, a spontaneous Rif^r derivative of NGR234. Conjugal matings were performed by mixing concentrated suspensions of the donor and recipient strains, spotting onto a 0.2-μm-pore-size cellulose acetate filter on a TY agar plate, and incubating for 18 h at 28°C. Following incubation, cells were removed from the filter and plated in dilution series on RMM plates supplemented with rifampin and kanamycin. Prototrophic Rif^r Km^r transconjugants were streak purified, and the integration of the plasmid into pNGR234a was verified by PCR with primers flanking the site of insertion.

Conjugal transfer assays. NGR234 with pCF429 carrying a Km^r marker integrated into the *traM* gene of pNGR234a was used as a conjugal donor. A plasmid expressing $lacI^q$, pSW213, was introduced into this background to allow subsequent introduction of the P_{lac}-*traR* plasmid pBDJ6 (see Results). The recipient was NGR234-ANU265, a derivative cured for pNGR234a. Likewise, *A. tumefaciens* C58 C1RS was also employed as a heterologous recipient. Cultures of donors and recipients were grown to an optical density at 600 nm (OD_{600}) of 1.0. Cells were collected by centrifugation at 18,000 × *g* and concentrated 20- to 100-fold in RMM. The donor cell suspensions were mixed with an equal volume of recipient cells, spotted onto 0.2-μm-pore-size cellulose acetate filters on unsupplemented RMM agar and on RMM agar supplemented with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and incubated at 28°C for 18 h. Cells were resuspended, and viable counts were performed for (i) transconjugants (Km^r Sm^r transconjugants for NGR234-ANU265 recipients and Km^r Sm^r Rif^r transconjugants for *A. tumefaciens* C58 C1RS recipients) and (ii) Km^r conjugal donors. Conjugation efficiencies were calculated as the ratio of transconjugants per recovered donor. Donor and transconjugant identities were further verified by randomly amplified polymorphic DNA PCR analysis with the short primers 5′-GTAGACCCGT and 5′-GGTGCGGGAA to generate genomic fingerprints (70).

Preparation of NGR234 culture extracts and bioassays for acyl HSLs. Crude preparations of acyl-HSLs were generated from whole cultures as described by Marketon et al. (43; M. M. Marketon and J. E. González, personal communication). NGR234 derivatives were cultivated to an OD_{600} of 1.5 in 5 ml of TY medium. An equal volume of dichloromethane was added to the cultures, and the mixtures were vortexed for 10 min with occasional venting. The vortexed extractions were centrifuged at 12,000 × *g* for 5 min to separate the aqueous and organic phases. The dichloromethane phase was collected and evaporated in a fume hood. The dried extract was dissolved in 200 μl of fresh dichloromethane. A control with autoclaved TY medium alone was performed in parallel.

Concentrated dichloromethane extracts (10 μl) were fractionated by reverse-phase thin-layer chromatography (TLC) with C_{18} silica plates (J.T. Baker, Phillipsburg, N.J.) in a mobile phase of 60% methanol as described previously (63). Following the TLC fractionation, the plates were air dried and acyl-HSLs were detected by overlaying with soft agar containing either *Chromobacterium violaceum* CV026 or *A. tumefaciens*(pCF218)(pCF372), both of which cannot produce but respond to acyl-HSLs (17, 45). *A. tumefaciens*(pCF218)(pCF372) activates expression of a *traI-lacZ* fusion (derived from the *A. tumefaciens* Ti plasmid *traI* gene and activated by the Ti plasmid TraR) in the presence of exogenous acyl-HSLs of variable chemistries ranging from C_6 to C_{12} in acyl chain length (74). Provision of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the overlay agar allows visualization of acyl-HSLs as spots of blue coloration after overnight incubation at 28°C. Likewise, *C. violaceum* CV026 cultured in Luria-Bertani agar produces the pigment violacein in response to acyl-HSLs ranging from C_4 to C_6, generating similar spots over the areas of the TLC plate with acyl-HSL fractions after incubation at 37°C (45). Standards of organically synthesized acyl-HSLs were fractionated alongside the extracts.

RNA isolation and primer extension assays. Total RNA was isolated from NGR234 cultures grown in RMM by using the technique of Summers (61). Primer extension reactions were performed with an estimated 30 μg of RNA, using avian myeloblastosis virus reverse transcriptase and [γ-^{32}P]-end-labeled oligonucleotides as described previously (17). The oligonucleotides 5′-TGGGT TCGTCGGGAGCGGTGG, 5′-GAGGCAGGTTTCTTCATTCCG, and 5′-AT GGCTGCGGAGAAGTTGGG were used for *traA*, *traC*, and *traI* transcripts, respectively. An oligonucleotide complementary to the *tetA* transcript encoded on pSW213 harbored in all of the NGR234 strains tested, 5′-CGATCAGGGG TATGTTGGGT, was used for control reactions. Reaction products were frac-

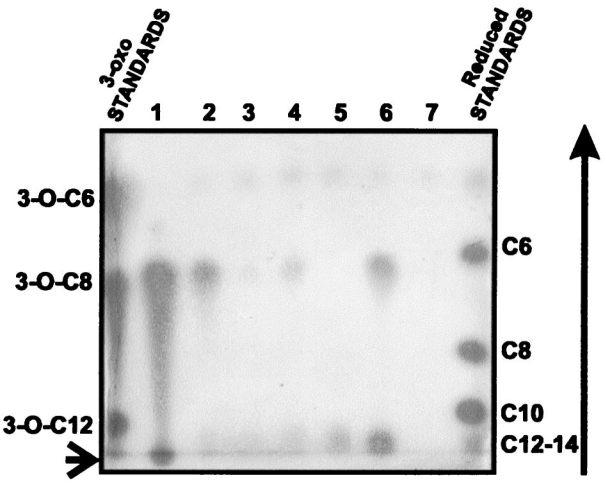

FIG. 2. Bioassay of whole-cell extracts from *Rhizobium* sp. strain NGR234 derivatives. TLC of dichloromethane whole-cell extracts from various NGR234 derivatives standardized to an OD_{600} of 1.5 and fractionated on C_{18} reverse-phase silica plates in a mobile phase of 60% methanol is shown. The plates were overlaid with acyl-HSL-responsive reporter strain *A. tumefaciens* NTL4(pCF218)(pCF372) as described previously (59, 74). Lane 1, NGR234(pSW213, *lacI*^{q}) (pBDJ6, P_{lac}-*traR*) diluted 200-fold. Lane 2, NGR234(pSW213). Lane 3, NGR234-XH100 (*traI* null pXH100 integrant). Lane 4, NGR234-XH100(pBDJ6, P_{lac}-*traR*). Lane 5, NGR234-ANU265 (pNGR234a cured). Lane 6, NGR234-ANU265(pBDJ6, P_{lac}-*traR*). Lane 7, extract of RMM. The vertical arrow indicates the direction of mobile phase flow, and the horizontal arrow marks the point of sample application. Synthetic standards flank sample lanes. Synthetic 3-oxo-acyl-HSL standards (3-O-C) are as follows: 3-oxo-C_6-HSL, 0.25 pmol; 3-oxo-C_8-HSL, 0.21 pmol; 3-oxo-C_{12}-HSL, 0.25 nmol. Synthetic acyl-HSL standards (C) are as follow: C_6-HSL, 0.26 nmol; C_8-HSL, 2.5 pmol; C_{10}-HSL, 2.5 nmol; C_{12}-HSL, 2.5 nmol; C_{14}-HSL, 2.5 nmol. The image was prepared with Corel Photopaint 9.0 and CorelDraw 9.0.

tionated on 6% polyacrylamide denaturing gels containing 7 M urea, and dried gels were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). A DNA sequencing ladder was obtained with the *traI*-specific oligonucleotide and pXH103 carrying the *traI* promoter region, using α-^{35}S-dATP, Sequenase enzyme, and the reaction conditions recommended by the enzyme supplier (United States Biochemical Corp.).

RESULTS

TraR and TraI from pNGR234a function as quorum-sensing regulators. Although the proteins encoded by the *traI*, *traR*, and *traM* genes on pNGR234a share sequence similarity with their counterparts on the *A. tumefaciens* Ti plasmids, there was not direct evidence for analogous function (12). Therefore, we analyzed the activity of these regulatory proteins in vivo.

To examine the acyl-HSLs produced by NGR234, dichloromethane extracts were prepared from whole NGR234 cultures grown in TY medium. Aliquots of the extracts were fractionated on reverse-phase TLC, and acyl HSLs were detected by overlaying the plates with acyl-HSL-responsive reporter strains. A single spot of activity, which comigrated with the synthetic 3-oxo-C8-HSL standard and also gave a typical tadpole conformation of the 3-oxo-acyl-HSLs, was detected using the *A. tumefaciens* reporter strain (Fig. 2, lane 2) (59). An additional faint, slowly migrating spot of activity was observed very close to the point of sample application. A very weak, rapidly migrating spot was observed in all samples, including the medium-only control (Fig. 2, lane 7). No activity was detected using the *C. violaceum* reporter with either the direct activation or inhibition assay (data not shown) (assay described in reference 45).

The *traR* gene was PCR amplified from NGR234 genomic DNA and used to construct a BHR P_{lac}-*traR* fusion plasmid (pBDJ6). Likewise, the *traI* promoter region was isolated and fused to *lacZ* carried on a separate, compatible BHR plasmid (pBDJ3). These two constructs were introduced into *A. tumefaciens* NTL4, a strain cured of the Ti plasmid and hence lacking the Ti-encoded quorum-sensing regulators. The addition of cell-free culture fluids containing 3-oxo-C8-HSL (from an overproducing *A. tumefaciens* derivative) induced *traI-lacZ* expression by over 3,000-fold in the *A. tumefaciens* derivative harboring P_{lac}-*traR* (Table 2). Expression in the absence of the acyl-HSL or the *traR*-expressing plasmid remained very low. Dose-response experiments comparing NGR234 *traI-lacZ* expression activated by NGR234 *traR* in the *A. tumefaciens* NTL4 background revealed that the NGR234-based system was very sensitive to 3-oxo-C8-HSL and was activated to high levels of expression (Fig. 3).

TraM inhibits TraR-dependent transcriptional activation. In *A. tumefaciens* the antiactivator TraM associates with TraR to form an inactive complex (30, 40, 62). Artificially elevated expression of *traM* results in a reduction of TraR-dependent activation of target promoters (13, 28). To determine whether the *traM* gene carried on pNGR234a has a similar inhibitory function, pDP101, in which *traR* and *traM* are expressed in tandem under the control of the P_{lac} promoter (P_{lac}-*traR-traM*), was constructed. An *A. tumefaciens* NTL4 derivative harboring the P_{lac}-*traR-traM* pDP101 plasmid demonstrated a

TABLE 2. Regulation of the pNGR234a P_{traI} promoter[a]

Reporter[c]	Plasmid[d]	Relevant genotype	β-Galactosidase sp act (Miller units)[b]	
			Without AAI	With AAI[e]
pBDJ3	pBBR1-MCS5	*traI-lacZ,* vector	9 (<0.1)	9 (<0.1)
pBDJ3	pBDJ6	*traI-lacZ* P_{lac}-*traR*	1 (0.1)	3,511 (235)
pBDJ3	pDP101	*traI-lacZ* P_{lac}-*traR-traM*	1 (<0.1)	70 (16)
pBDJ7	pBDJ6	*tra* box fill-in, P_{lac}-*traR*	4 (1.7)	25 (14)

[a] All strains were derived from Ti plasmidless *A. tumefaciens* NTL4.
[b] Results are averages from assays in triplicate with standard deviations in parentheses.
[c] pBDJ3, P_{traI}-*lacZ*; pBDJ7, *tra* box BamHI fill-in P_{traI}-*lacZ.*
[d] pBBR1-MCS5, vector control; pBDJ6, P_{lac}-*traR.*
[e] With 0.5% (vol/vol) cell-free supernatant of the AAI overproducer *A. tumefaciens* KYC6(pCF218), having bioactivity equivalent to 4 μM synthetic 3-oxo-C8-HSL.

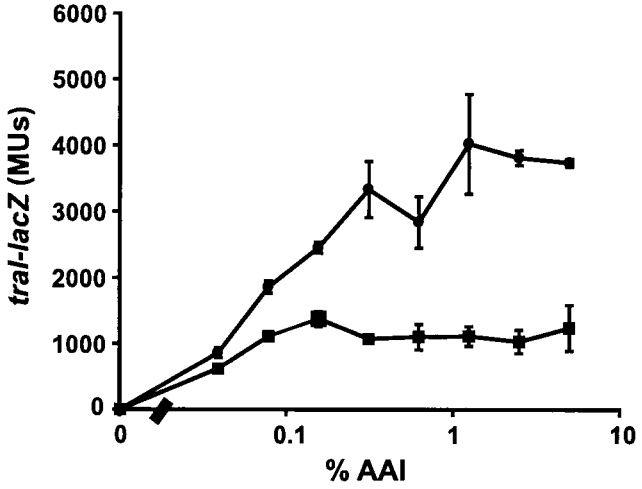

FIG. 3. Acyl-HSL dose-response curves. A comparison of dose-responsive expression of P_{traI}-*lacZ* fusions activated by TraR from *A. tumefaciens* NTL4 expressing (i) TraR (pBDJ6, P_{lac}-*traR*) and a P_{traI}-*lacZ* fusion (pBDJ3) from pNGR234a (circles) and (ii) TraR (pCF218, P_{tetR}-*traR*) and a P_{traI}-*lacZ* fusion (pCF372) from the *A. tumefaciens* Ti plasmid pTiR10 (squares) is shown. The concentration of a crude preparation of AAI (3-oxo-C8-HSL) from *A. tumefaciens* KYC6(pCF218) added at the time of culture inoculation is indicated as a percentage (volume/volume) (ranging from 0.48 to 48 μM equivalent bioactivity). Expression of *traI-lacZ* was measured in Miller units (MUs). Values are averages from assays performed in triplicate, and error bars indicate standard deviations.

50-fold reduction in AAI-dependent induction of the *traI-lacZ* fusion relative to pBDJ6 expressing *traR* alone (Table 2).

Ectopic expression of *traR* elevates *traI* expression on pNGR234a. To evaluate the expression of the *traI-lacZ* fusion in its parental background, pBDJ3 was introduced into NGR234. Only weak constitutive expression which was unaffected by the addition of saturating amounts of AAI was detectable (data not shown). In *A. tumefaciens* a similar pattern of expression is observed for TraR target genes due to tight regulation of *traR* expression by crown gall tumor-released opines (Fig. 1A) (18, 52). Therefore, it seemed likely that the activation of the plasmid-borne *traI-lacZ* fusion was similarly limited by the expression level of the native copy of *traR* on pNGR234a.

To test this hypothesis, a plasmid carrying an internal fragment of the *traI* coding sequence (pXH100) fused to a promoterless *lacZ* (*traI*::*lacZ*) was integrated by single crossover into pNGR234a to generate NGR234-XH100, resulting in a *traI* null mutation. Likewise, a plasmid carrying a larger fragment of *traI* with a complete 5′ end (pXH101) but fused to *lacZ* at the same point as in pXH100 was used to generate NGR234-XH101, retaining an intact, functional copy of *traI*. NGR234-XH100 expressed the integrated *traI*::*lacZ* weakly, and this was unaffected by addition of saturating AAI (Table 3). Surprisingly, introduction of the P_{lac}-*traR* plasmid pBDJ6 into NGR234-XH100 resulted in modest activation of the *traI*::*lacZ* fusion (basal level of 2 U elevated to 55 U). Addition of 0.1% (vol/vol) AAI to this culture resulted in strong induction of the *traI*::*lacZ* fusion. The P_{lac}-*traR* can be introduced stably into the NGR234-XH101 derivative (retaining the functional copy of *traI*) only when the strain harbors a *lacI*^{q} repressor-expressing plasmid, pSW213 (*lacI*^{q} is required to obtain transformants with the P_{lac}-*traR* plasmid in most NGR234 derivatives [see below]). The presence of *lacI*^{q} also provides for regulated expression of *traR* through IPTG. Introduction of the P_{lac}-*traR* plasmid into NGR234-XH101(pSW213) in the absence of IPTG resulted in very weak elevation of *traI*::*lacZ* expression, which was only marginally enhanced by addition of exogenous AAI (Table 3). However, addition of IPTG alone strongly induced *traI*::*lacZ* expression, and this was enhanced by exogenous AAI. These observations suggest that *traR* expression is limiting in wild-type NGR234 and in the strain NGR234-XH100, in which *lacI*^{q} represses expression of the pBDJ6-borne P_{lac}-*traR*.

Analysis of culture extracts reveals additional acyl-HSLs in NGR234. The presence of the P_{lac}-*traR* plasmid strongly elevated expression of the pNGR234a *traI* gene. Consistent with this observation, the presence of the P_{lac}-*traR* plasmid in an otherwise wild-type NGR234 harboring the *lacI*^{q} plasmid pSW213 and induced with IPTG resulted in strong overproduction of an acyl-HSL activity as assessed by cross-feeding assays with the acyl-HSL-responsive *A. tumefaciens* reporter strain (data not shown). TLC bioassay analysis of dichloromethane extracts from cultures of the NGR234 strain revealed overproduction of a compound that comigrated with the 3-oxo-C8-HSL standard and with the compound from untransformed NGR234 (Fig. 2, lanes 1 and 2 [the sample in lane 1 was 200-fold more dilute than the other samples]). In addition, a spot of activity that did not migrate far from the point of sample application was readily visible. Surprisingly, extracts from the *traI* null mutant (NGR234-XH100), although significantly reduced for activity, possessed two weak inducing activities, one that comigrated with the 3-oxo-C8-HSL standard

TABLE 3. TraR is limiting for *traI* activation in NGR234

Strain[a]	Additional plasmid(s)	Relevant genotype	β-Galactosidase sp act (Miller units)[b]			
			Without IPTG		With IPTG (0.1 mM)	
			Without AAI	With AAI[c]	Without AAI	With AAI
NGR234-XH100	None	*traI* null, *traI*::*lacZ*	2 (<1)	2 (<1)	ND[d]	ND
NGR234-XH100	pBDJ6	*traI* null, *traI*::*lacZ* P_{lac}-*traR*	55 (12)	756 (162)	ND	ND
NGR234-XH101	pBDJ6, pSW213	*traI* wild type, *traI*::*lacZ* P_{lac}-*traR lacI*^{q}	18 (4)	27 (5)	642 (55)	952 (14)

[a] All strains were derived from NGR234.
[b] Results are averages from assays in triplicate, with standard deviations in parentheses.
[c] With 0.1% (vol/vol) cell-free supernatant of the AAI overproducer *A. tumefaciens* KYC6(pCF218), having bioactivity equivalent to 0.8 μM synthetic 3-oxo-C8-HSL.
[d] ND, not determined.

and a second that migrated only slightly from the application site (Fig. 2, lane 3). Extracts from NGR234-XH100 harboring the P_{lac}-*traR* plasmid contained these two activities, both notably stronger than that from NGR234-XH100 alone (Fig. 2, lanes 3 and 4). This observation suggested that in addition to the signal molecules specified by *traI* carried on pNGR234a, a second acyl-HSL activity that comigrates with 3-oxo-C8-HSL is synthesized by NGR234. Consistent with this, extracts from NGR234-ANU265, a strain that is cured of pNGR234a, contained a barely detectable activity comigrating with 3-oxo-C8-HSL. The slowly migrating activity appeared to be slightly more abundant in NGR234-ANU265 than in strains harboring pNGR234a (Fig. 2, lane 5). Introduction of the P_{lac}-*traR* into the NGR234-ANU265 strain resulted in extracts that were again significantly elevated for both activities (Fig. 2, lane 6).

TraR activates pNGR234a promoters associated with putative *tra* box-type elements. Our findings suggested that NGR234 P_{traI} is regulated by its cognate TraR and acyl-HSL. Careful inspection of the NGR234 sequence revealed potential regulatory sites in positions highly analogous to those of the Ti plasmid *tra* boxes (Fig. 1B). Directly upstream of the NGR234 *traI* translational start site is an 18-bp inverted repeat, tentatively designated NGR *tra* box II, with similarity to *tra* box elements identified in *A. tumefaciens* (Fig. 1B). A sequence 542 bp further upstream of *traI* has significant similarity to NGR *tra* box II and is tentatively designated NGR *tra* box III. Within the pNGR234a *traA-traC* intergenic region in the Dtr-*oriT* cluster, a single possible *tra* box promoter element, presumptively designated NGR *tra* box I, is positioned identically to *tra* box I on the *A. tumefaciens* Ti plasmids (17). The primary sequence conservation between the presumptive pNGR234a sequence elements and the Ti plasmid *tra* boxes is modest, and there is surprising sequence degeneracy among the NGR234 elements (averaging 55% identity) compared to the similarity between Ti plasmid *tra* boxes (averaging 88%).

A derivative of pBDJ3 (carrying the *traI-lacZ* fusion) designated pBDJ7 carries a 4-bp insertion in the exact center of the presumptive NGR *tra* box II sequence (underlined in Fig. 1B). *A. tumefaciens* NTL4 harboring pBDJ7 and the P_{lac}-*traR* plasmid pBDJ6 was 500-fold reduced for *traI-lacZ* expression in the presence of 3-oxo-C8-HSL relative to pBDJ3 with the intact *tra* box (Table 2).

Primer extension assays to detect the presumptive transcripts initiating from P_{traI}, P_{traA}, and P_{traC} promoters were performed on total RNA preparations of NGR234 harboring the pSW213 plasmid to allow *lacI*^{q}-dependent control of P_{lac} and either pBDJ6 (P_{lac}-*traR*) or the pBBR1-MCS5 vector control. Reverse transcripts specified by the *traI* and *traC* promoters were detected in the RNA preparation from NGR234 (pBDJ6)(pSW213) grown in the presence of IPTG to induce P_{lac}-*traR* expression but were absent in all other RNA preparations (Fig. 4). No detectable primer extension products were specified by the *traA* primer for any of the RNA preparations. Three different *traA* antisense primers were tested, and none yielded a detectable reverse transcript. A primer extension product for the *tetA* gene carried on pSW213 was detectable in all RNA preparations and served as a control. These results suggested that although the *tra* operons initiated with *traI* and *traC* (Mpf and Dtr, respectively) are strongly activated by TraR, the Dtr operon initiated by *traA* may not be significantly expressed under these conditions.

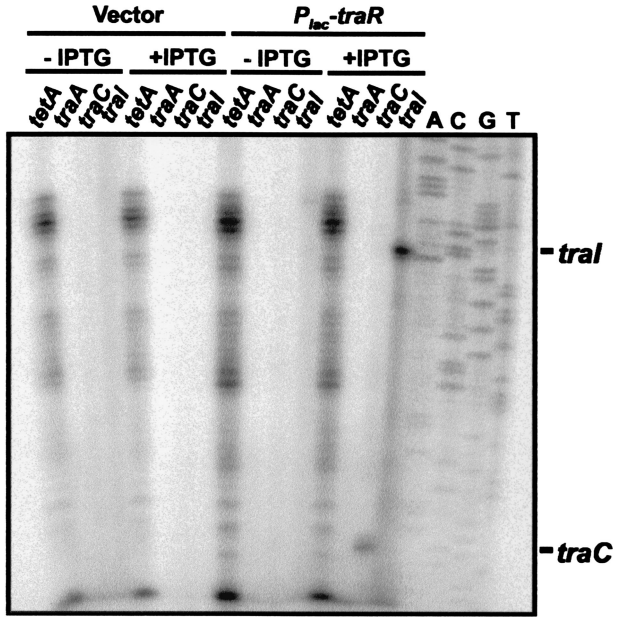

FIG. 4. Expression of pNGR234a *tra* genes. Total RNA was isolated from cultures of NGR234(pSW213, *lacI*^{q}) harboring either pBBR1-MCS5 as a vector control or pBDJ6 (P_{lac}-*traR*) and grown in RMM either alone or supplemented with 0.5 mM IPTG. Primer extensions were performed on 30 μg of RNA with avian myeloblastosis virus reverse transcriptase and oligonucleotides complementary to the predicted 5′ coding sequences of *traA*, *traC*, and *traI* of pNGR234a and *tetA* encoded by pSW213. An ^{35}S-labeled DNA sequencing ladder generated with the *traI* primer and pXH103 carrying the promoter sequences upstream of *traI* on pNGR234a was used to locate the *traI* TSP and to predict the *traC* TSP. Reaction products were fractionated on a 6% denaturing polyacrylamide gel with 7 M urea, and the dried gel was analyzed with a PhosphorImager. The image was prepared with Corel Photopaint 9.0 and CorelDraw 9.0.

The primer extension product for *traI* was used to map its transcription start point (TSP) by comparison to a sequencing reaction with the *traI*-specific oligonucleotide (Fig. 4). The *traI* TSP was identified 24 bp upstream of the predicted *traI* start codon, with NGR *tra* box II centered at position −44 (Fig. 1B). The size of the *traC* reverse transcript, based on the *traI* sequencing ladder, predicted a TSP 34 bp upstream of the *traC* start codon, with the presumptive NGR *tra* box I centered at position −46 (Fig. 1B). Although no primer extension product was observed for the transcript initiating with *traA*, the position of NGR *tra* box I would predict a TSP 129 bp upstream of the *traA* start codon and upstream of the predicted *oriT* sequence (Fig. 1B).

Quorum-sensing regulators of *A. tumefaciens* and NGR234 do not cross-function. The TraR and TraI proteins encoded on pNGR234a share significantly higher similarity with TraR and TraI from the Ti plasmids (56 and 27% identity with regulators from the octopine-type Ti plasmids, respectively) than they do with other LuxR and LuxI homologues. Additionally, the organization of the pNGR234a *tra-rep* cluster clearly indicates that these plasmid-borne systems are orthologous, perhaps reflecting a relatively recent ancestral plasmid replicon. Given

TABLE 4. In vivo assays of *traR* cross-functionality[a]

TraR plasmid	*traI-lacZ* fusion	β-Galactosidase sp act (Miller units)[b]	
		Without AAI	With AAI[c]
pCF218 (Ti *traR*)	pCF372 (Ti *traI*)	<1	1,437
	pBDJ3 (NGR234 *traI*)	13	15
pBDJ6 (NGR234 *traR*)	pCF372 (Ti *traI*)	2	<1
	pBDJ3 (NGR234 *traI*)	15	637

[a] All assays were performed with *A. tumefaciens* NTL4.
[b] Results are averages from assays in triplicate.
[c] With 5% (vol/vol) cell-free supernatant of the AAI overproducer *A. tumefaciens* KYC6(pCF218), having bioactivity equivalent to 40 μM synthetic 3-oxo-C8-HSL.

TABLE 5. In vivo assays of *traM* cross-functionality[a]

TraM Plasmid	β-Galactosidase sp act (Miller units)[b] with:			
	No inducer	AAI[c]	IPTG (1 mM)	IPTG + AAI
pBBR1-MCS5 (vector)[d]	<5	571	<5	551
pGB200 (Ti *traM*)	<5	528	<5	32
pCF424 (NGR234 *traM*)	<5	578	<5	612

[a] All assays were performed with *A. tumefaciens* NTL4 harboring pCF218 (P_{tetR}-*traR*) and pCF372 (P_{traI}-*lacZ*).
[b] Results are averages from assays in triplicate.
[c] With 0.05% (vol/vol) cell-free supernatant of the AAI overproducer *A. tumefaciens* KYC6(pCF218), having bioactivity equivalent to 0.4 μM synthetic 3-oxo-C8-HSL.
[d] Both *traM* expression plasmids are derivatives of pBBR1-MCS5.

these observations, it seemed plausible that the quorum-sensing components might cross-function. This is apparently correct for the TraI orthologues, where the primary reaction product of NGR234 TraI is likely to be 3-oxo-C8-HSL. To test for TraR protein cross-functionality, the pBDJ6 (P_{lac}-*traR*) plasmid was introduced into *A. tumefaciens* NTL4 harboring pCF372, carrying the Ti plasmid *traI-lacZ* fusion. Conversely, pCF218, strongly expressing the Ti plasmid TraR, was introduced into *A. tumefaciens* NTL4 harboring pBDJ3, carrying the NGR234 *traI-lacZ* fusion. Neither *traI-lacZ* fusion was activated by the noncognate TraR, although each was strongly activated by its cognate regulator in the presence of AAI (Table 4).

The NGR234 *traM* gene was tested for its ability to inhibit transcriptional activation by the Ti plasmid TraR, as it does with the NGR234 TraR. *A. tumefaciens* harboring the Ti plasmid *traR* expression plasmid pCF218 and the *traI-lacZ* fusion on pCF372 was transformed with pCF424, carrying the NGR234 *traM* expressed as a P_{lac}-*traM* fusion. A control strain harboring pGB200, expressing the Ti plasmid TraM but otherwise identical to pCF424, was also analyzed. As expected, when its expression was induced with IPTG, the Ti plasmid *traM* strongly inhibited Ti plasmid *traR* activation of *traI-lacZ* expression (Table 5). However, expression of the NGR234 *traM* in this background had no effect on the activity of the Ti plasmid *traR* and was identical to that of a strain lacking either P_{lac}-*traM* plasmid.

Low-efficiency conjugal transfer of pNGR234a is not improved by ectopic expression of *traR*. The effect of TraR activation on conjugal transfer of pNGR234a to the NGR234-ANU265 recipient (pNGR234a cured) was examined. The NGR234 strain harboring the P_{lac}-*traR* derivative repressed by *lacI*^{q} can be grown in the presence of IPTG, and although the inducer slows growth (see below), it also strongly elevates pNGR234a *traI* expression and acyl-HSL synthesis. A pNGR234a derivative carrying a Km^r marker (NGR234-BDJ3) conjugated at an extremely low efficiency (approximately 10^{-9}), and this remained constant regardless of the presence of the P_{lac}-*traR* plasmid and the IPTG inducer (Table 6). Analysis of the putative transconjugants with pNGR234a-specific primers revealed the presence of the plasmid (data not shown). As NGR234-ANU265 transconjugants harboring pNGR234a would be virtually indistinguishable from the donor strain, it was possible that the infrequent Km^r colonies were spontaneous Km^r donors. Conjugation assays with *A. tumefaciens* C58 C1RS, a strain that lacks the Ti plasmid, were performed (8). Efficiency of transfer of the Km^r marker to C58 C1RS from NGR234-BDJ3 was equivalent to that when NGR234-ANU265 was used as a recipient (Table 6). Randomly amplified polymorphic DNA PCR analysis of the C58 C1RS transconjugants demonstrated that the transconjugants were C58 C1RS derivatives that had received the pNGR234a plasmid (data not shown).

Subsequent use of the C58 C1RS harboring the Km^r-marked pNGR234a as a donor with the plasmid-cured NGR234-ANU265 as a recipient also resulted in similarly low conjugal transfer efficiencies, suggesting that the weak transfer observed

TABLE 6. Conjugal transfer of pNGR234a

Donor strain[a]	Recipient[b]	Transconjugants per donor[c]	
		Without IPTG	With IPTG (0.5 mM)
NGR234-BDJ3(pBBR1-MCS5, vector)	NGR234-ANU265	1×10^{-8}	4×10^{-9}
NGR234-BDJ3(pBDJ6, $P_{lac\text{-}traR}$)	NGR234-ANU265	4×10^{-9}	6×10^{-9}
NGR234-BDJ3(pBBR1-MCS5, vector)	C58 C1RS	ND[d]	4×10^{-9}
NGR234-BDJ3(pBDJ6, $P_{lac\text{-}traR}$)	C58 C1RS	ND	2×10^{-9}

[a] NGR234-BDJ3 carries a plasmid insertion of pCF429, carrying Km^r integrated into the *traM* gene on pNGR234a. All strains also harbor the pSW213 plasmid expressing *lacI*^{q}.
[b] Conjugal transfer assays were performed as described in Materials and Methods with approximately 10^{10} recipient cells per mating (either NGR234-ANU265 or *A. tumefaciens* C58 C1RS).
[c] Conjugal efficiency calculated as the number of Km^r Sm^r transconjugants per output conjugal donor for NGR234-ANU265 and the number of Km^r Sm^r Rif^r transconjugants per output conjugal donor for *A. tumefaciens* C58 C1RS.
[d] ND, not determined.

TABLE 7. Transformation of a *traR* overexpression plasmid

Recipient	Resident plasmid	Transformed plasmid	Transformants/μg[a] with:	
			No inducer	Synthetic 3-oxo-C8-HSL (20 nM)
NGR234(pNGR234a)	pSW172 (no *lacI*^{q})	pBBR1-MCS5	5.0×10^4	ND[b]
		pBDJ6 (P_{lac}-*traR*)	<3.0[c]	ND
	pSW213 (*lacI*^{q})	pBBR1-MCS5	4.3×10^4	ND
		pBDJ6 (P_{lac}-*traR*)	4.2×10^4	ND
NGR234-XH100 (*traI* null)	None	pBBR-MCS5	4.6×10^4	4.4×10^4
		pBDJ6 (P_{lac}-*traR*)	5.3×10^4	<3.0[c]
NGR234(pNGR234a)	pSW172 (no *lacI*^{q})	pDP101 (P_{lac}-*traR-traM*)	3.0×10^4	ND
	pSW213 (*lacI*^{q})	pDP101 (P_{lac}-*traR-traM*)	1.0×10^5	ND
NGR234-ANU265	None	pBBR1-MCS5	3.3×10^4	3.7×10^4
		pBDJ6 (P_{lac}-*traR*)	4.1×10^4	<3.0[c]
C58 C1RS(pNGR234a-BDJ3)	None	pBBR1-MCS5	6.0×10^6	ND
		pBDJ6 (P_{lac}-*traR*)	8.0×10^6	ND

[a] Gm^r transformants were enumerated following transformation with 350 ng of plasmid.
[b] ND, not determined.
[c] There were zero transformants with 350 ng of plasmid.

is a characteristic intrinsic to pNGR234a and is not unique to the NGR234 genetic background (data not shown).

Ectopic expression of *traR* reduces the growth rate of NGR234. We repeatedly were unable to introduce pBDJ6 (P_{lac}-*traR*) into a wild-type NGR234 strain by electroporation. In related rhizobia, quorum sensing significantly affects growth rate (24, 58). We reasoned that the introduction of the strongly expressed P_{lac}-*traR* might be growth inhibitory, preventing establishment of the plasmid. Two derivatives of NGR234 were constructed to examine this possibility: one harboring pSW213, expressing the *lacI*^{q} repressor of P_{lac}, and the other harboring pSW172, which is identical to pSW213 but without the *lacI*^{q} gene (4). The NGR234 strain harboring pSW213 would have relatively high levels of LacI, repressing P_{lac}-*traR* expression. The efficiency of electroporation of pBDJ6 (P_{lac}-*traR*) into the NGR234(pSW213) *lacI*^{q}-expressing strain and the control recipient without *lacI*^{q} [NGR234(pSW172)] was compared with that of a pBBR1-MCS5 vector control. Efficient transformation (4.3×10^4 transformants/μg plasmid) into the derivative harboring *lacI*^{q} was observed for pBDJ6, but, consistent with previous results, no transformants were observed for transformation of pBDJ6 into the control background without *lacI*^{q} (Table 7). In contrast, the pBBR1-MCS5 vector control was transformed with equal efficiency into both recipient strains (Table 7). Null mutation of the pNGR234a *traI* gene (NGR234-XH100) resulted in a strain that was transformed with the P_{lac}-*traR* plasmid at an efficiency similar to that of the vector control (Table 7). Addition of synthetic 3-oxo-C8-HSL to 100 nM prior to or immediately following electroporation prevented transformation of pBDJ6 into the *traI* null mutant but did not affect the vector control. Collectively, these results indicate that transformation inhibition requires expression of the *traR* gene and the presence of its inducing ligand. We designated this phenotype TraR-mediated transformation inhibition (Tmi).

The *lacI*^{q}-expressing NGR234 strain harboring the P_{lac}-*traR* expression plasmid pBDJ6 grew as normal-size colonies and had a cellular morphology typical for NGR234 (data not shown). However, NGR234(pSW213 *lacI*^{q}) harboring pBDJ6 had a generation time of 3.5 h/doubling in ATGN broth cultures, compared to that of 2 h for the same strain with the pBBR1-MCS5 vector control (Fig. 5). Addition of IPTG to induce expression of P_{lac}-*traR* increased the generation time to 6 h/doubling for the strain harboring pBDJ6 but did not affect the pBBR1-MCS5 vector control. The final culture density of the IPTG-induced, growth rate-inhibited culture did eventually reach an optical density similar to that of the others, indicating a reduction in growth rate but not early entry into stationary phase. The *traI* null mutant NGR234-XH100 harboring pBDJ6 (P_{lac}-*traR*) was observed to grow similarly to the wild-type strain (2 h/doubling). However, addition of synthetic 3-oxo-C8-HSL substantially reduced the growth rate of this strain, to 14 h/doubling at high concentrations of the inducer (100 nM), while the growth rate of the *traI* null mutant with the pBBR1-MCS5 control was unaffected. These data suggest that ectopic expression of *traR* in the presence of its inducing ligand results in a slowing of growth in NGR234.

The TraM and TraR proteins of *A. tumefaciens* form an

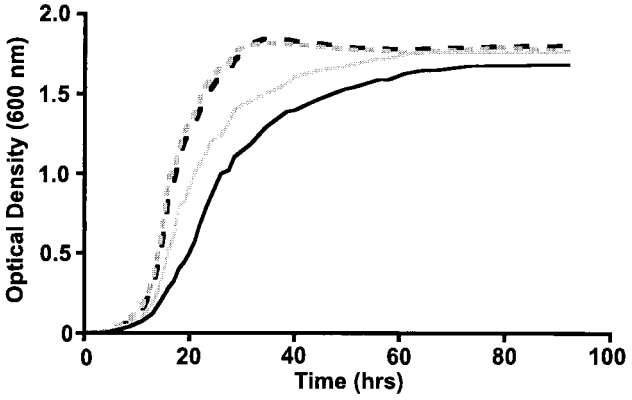

FIG. 5. Growth rate inhibition of NGR234. Growth curves for NGR234 derivatives cultivated in RMM over a 4-day incubation period are shown. Dashed lines, NGR234(pSW213)(pBBR1-MCS5) control; solid lines, NGR234(pSW213)(pBDJ6, P_{lac}-*traR*). Cultures were grown in RMM plus gentamicin and tetracycline (gray curves) and the identical medium supplemented with 1 mM IPTG (black curves).

antiactivation complex that prevents TraR from activating transcription of Ti plasmid target genes (30, 40, 62). Reasoning that the growth inhibition was likely due to the activation of TraR target gene expression, we examined transformation with a plasmid expressing the NGR234 *traR* and *traM* genes from the same promoter (pDP101, P_{lac}-*traR-traM*). Transformation of pDP101 was observed in both the *lacI*^{q} and control backgrounds, although the efficiency was improved in the *lacI*^{q}-harboring strain (Table 7). This result suggests that inhibition of TraR, either at the level of gene expression, as with the *lacI*^{q} recipient, or posttranslationally by antiactivation with TraM effectively suppresses the Tmi phenotype.

Growth rate inhibition requires *traR* and *traI* from pNGR234a and functions encoded elsewhere in the genome. Introduction of the P_{lac}-*traR* plasmid pBDJ6 was examined in NGR234-ANU265, which lacks pNGR234a. Transformation of pBDJ6 was equally efficient to that of the vector control when NGR234-ANU265 was used as a recipient (Table 7). However, this strain lacks the pNGR234a *traI* gene and therefore synthesizes only trace levels of what is presumptively 3-oxo-C8-HSL (Fig. 2). Preincubation of recipient NGR234-ANU265 with 100 nM synthetic 3-oxo-C8-HSL prevented transformation with the P_{lac}-*traR* plasmid, while transformation with a vector control was unaffected (Table 7). Furthermore, the growth rate of NGR234-ANU265 harboring pBDJ6 was significantly inhibited by addition of 3-oxo-C8-HSL, with 20 nM 3-oxo-C8-HSL increasing the generation time to 13 to 14 h/doubling.

A derivative of pNGR234a carrying a Km^r marker (pNGR234a::pCF429) was conjugally transferred into the Ti plasmid-cured *A. tumefaciens* derivative C58 C1RS. Subsequent introduction of the P_{lac}-*traR* plasmid (pBDJ6) was as efficient as that of the vector control (Table 7), and the growth rate of the pBDJ6 transformant was normal, suggesting that growth inhibition requires additional functions encoded specifically within the NGR234 genome.

DISCUSSION

Quorum-sensing regulators and signals in NGR234. In this study, we demonstrate that the pNGR234a TraI, TraR, and TraM orthologues function similarly to those of the well-characterized Ti plasmid quorum-sensing regulators (71). Although we have not chemically characterized the product of the pNGR234a TraI, its fractionation characteristics in the TLC-based bioassay suggest that it is 3-oxo-C8-HSL. The pNGR234a *traI* null mutant (NGR234-XH100) has significantly reduced synthesis of this activity (Fig. 2). Surprisingly, the *traI* null mutant and a pNGR234a-cured derivative synthesized low levels of an inducing activity that comigrated with 3-oxo-C8-HSL and was elevated by TraR (Fig. 2). These findings are also consistent with our observation that introduction of a P_{lac}-*traR* plasmid into the pNGR234a *traI* null mutant derivative modestly elevated expression of an integrated *traI*::*lacZ* fusion in the absence of exogenous AAI, presumably due to the low levels of the signal molecule in this strain (Table 3). The putative signal molecule is likely to be 3-oxo-C8-HSL but may be 3-hydroxy-C8-HSL, as our TLC assay would not readily distinguish between low levels of these compounds. Synthesis of the inducing signal must require an enzymatic activity in addition to TraI, perhaps a second *traI*-like enzyme, encoded on the chromosome or pNGR234b. Expression of this gene may be weakly activated by the pNGR234a TraR protein (see tentative model in Fig. 6). Partial analysis of the ANU265 genome has not identified a second acyl-HSL synthase (65).

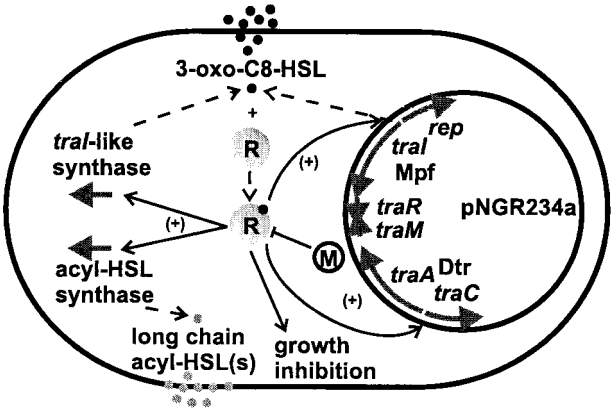

FIG. 6. Model of quorum-sensing gene regulation in NGR234. A tentative model for quorum sensing in a single cell of NGR234, based on the findings presented in this study, is shown. Genes and operons are represented as gray arrows (the indicated genes and operons are not to scale). Solid lines with arrowheads, known or suspected regulatory pathways (parenthetical pluses indicate positive regulation); dashed lines with arrowheads, synthesis of acyl-HSLs or interaction of acyl-HSLs with TraR. Small filled circles are acyl-HSLs. Circles labeled with R and M represent TraR and TraM, respectively. The solid line ending in the bar reflects inhibition of TraR by TraM.

A more nonpolar activity, migrating as slowly as our C_{12}/C_{14}-HSL standards, was detected in all extracts analyzed. The same activity was also detectable in extracts of cell-free culture fluids (data not shown) but was notably more abundant in whole-cell extracts, consistent with a long-chain acyl-HSL (43). Synthesis of this putative long-chain acyl-HSL was elevated in the absence of pNGR234a and further induced by pNGR234a TraR (Fig. 2). It is possible that this compound(s) is synthesized by the same enzyme responsible for the pNGR234a-independent synthesis of the more polar activity. However, *traI* orthologues have not been reported to synthesize long-chain acyl-HSLs, and it is equally plausible that this activity is synthesized by a different enzyme encoded elsewhere in the NGR234 genome (Fig. 6). Several other rhizobia synthesize long-chain acyl-HSLs via the activity of LuxI homologues (7, 37, 42). In *R. leguminosarum* the CinI protein directs synthesis of *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (3-OH-$C_{14:1}$-HSL), and this compound has a growth-inhibitory activity (24, 58). Furthermore, the presence of the symbiotic plasmid pRL1JI represses but does not abolish 3-OH-$C_{14:1}$-HSL synthesis, consistent with our observations that the pNGR234a-cured strain produces higher levels of the slowly migrating activity than the strain harboring pNGR234a.

Expression of *traR* is limiting in NGR234. The pNGR234a TraR protein responds to 3-oxo-C8-HSL to activate at least two of the *tra-trb* structural gene operons (Dtr and Mpf) on the symbiotic plasmid (Fig. 6). Activation of the promoter upstream of *traI* is highly sensitive to 3-oxo-C8-HSL. However, it is striking that activation of *tra* genes on pNGR234a occurs only when *traR* is expressed ectopically. Our results suggest

that expression of the pNGR234a *traR* gene is limiting, similar to the case for the *A. tumefaciens* Ti plasmid-borne *traR* genes (18, 52). Expression of the Ti plasmid *traR* gene, and hence conjugal transfer of the Ti plasmid, is strictly regulated by the presence of a specific conjugation-inducing subset of opines, compounds released from the crown gall tumors of infected plants (22, 33). We speculate that an analogous upstream signal cascade regulates pNGR234a *traR* expression under a specific set of conditions, perhaps in response to plant metabolites. Although legumes nodulated by rhizobia are not reported to produce opines per se, strains of the species *R. leguminosarum* and *S. meliloti* synthesize a class of inositol derivatives called rhizopines within nodules, and these are thought to feed free-living rhizobia in the rhizosphere (48). Rhizopine catabolism is relatively common among soil bacteria, and NGR234 has a cluster of genes homologous to the *moc* rhizopine catabolic genes of *S. meliloti*, suggesting that NGR234 may utilize these compounds (21, 65, 67). It is plausible that rhizopines regulate *traR* expression, similar to opines in *A. tumefaciens*. Alternatively, additional plant-released compounds or other aspects of the environment may control pNGR234a *traR* expression.

Regulation of pNGR234a genes by TraR and TraM. TraR activation of P_{traI} requires the NGR *tra* box II. Other putative NGR *tra* box sequences similar to this element but only weakly similar to Ti plasmid *tra* boxes were identified in positions almost identical to the positions of *tra* boxes on Ti plasmids (17). We provide direct evidence that TraR activates expression of the Dtr transcript initiating with *traC*, suggesting that *tra* box I is at least partially functional. However, despite repeated efforts we could not detect a transcript for the operon initiating with *traA*, suggesting that this operon is not activated by TraR or also requires additional factors.

Weak expression of the *traA* Dtr operon may explain why conjugal transfer of the pNGR234a plasmid is not enhanced by ectopic expression of *traR* in NGR234 (Table 6), although the *traI* and *traC* operons are strongly induced. The *traAFBH* operon encodes homologues of the nickase-helicase TraA and the peptidase TraF, both of which are essential for conjugal transfer of Ti plasmids (10). The rare transfer events that we observe may be due to weak basal expression of the pNGR234a *traAFBH* operon, or the plasmid may be mobilized in *trans* by other, unidentified NGR234 Dtr functions. The dynamic structure of the NGR243 genome, with reversibly integrated pNGR234a and pNGR234b episomes in equilibrium, could also contribute to the low conjugal transfer efficiency we observe for pNGR234a (11).

In *A. tumefaciens* TraR elevates plasmid copy number through activation of the *repABC* genes, which is mediated in part by interactions with *tra* box III immediately upstream of the *rep* operon and divergent from *traI* (36; S. C. Winans, personal communication). We have identified a sequence with weak similarity to NGR *tra* box II in a similar position relative to the pNGR234a *repABC* genes (a putative NGR *tra* box III), and we speculate that TraR influences the copy number of this symbiotic plasmid as well.

Ti plasmid-encoded TraR is strongly inhibited by formation of an antiactivation complex with TraM, and this inhibition is required for cell density responsiveness (13, 53, 55, 62). Our results suggest that the pNGR234a *traM* gene product is orthologous and inhibits the activity of TraR. Repeated attempts to generate a *traM* null mutant were unsuccessful, perhaps due to the growth-inhibitory affect of unregulated TraR activity (B. D. Jones and C. Fuqua, unpublished data). Therefore, we do not know whether this gene is required to maintain quorum-sensing functions suppressed under noninducing conditions, as is the Ti plasmid *traM*.

Functional divergence of the pNGR234a and Ti plasmid quorum-sensing regulators. Despite significant sequence conservation and similar mechanisms, the TraR and TraM quorum-sensing regulators from Ti plasmids and from pNGR234a do not cross-function when coexpressed. The overall levels of identity between these proteins are 27 and 19%, for TraR and TraM, respectively. However, alanine-scanning mutagenesis of *traM* carried on the octopine-type Ti plasmid, based on conservation with the pNGR234a TraM, revealed that 13 of the 18 conserved residues were required for TraM activity (62). Clearly, these quorum-sensing proteins are orthologous and share similar mechanisms but must recognize specific attributes of their cognate TraR proteins. The pNGR234a TraR responds to the same inducing ligand as the Ti plasmid TraR, but these proteins cannot recognize the DNA binding sites of the other protein, reflecting the divergence of their *tra* box sequences. LasR of *Pseudomonas aeruginosa* and LuxR of *Vibrio fischeri* are more divergent and productively recognize each other's DNA binding sites (23).

Growth inhibition and quorum sensing in NGR234. We observe a significant effect of TraR activity on the growth rate of NGR234, manifested most dramatically by the Tmi phenotype, i.e., the inability to transform wild-type NGR234 with a plasmid that strongly expresses *traR*. The Tmi phenotype is consistently associated with growth rate reduction, and we believe that these phenomena are manifestations of the same regulatory mechanism. Freshly electroporated cells provided with an extended incubation period in the absence of antibiotic selection produce slow-growing P_{lac}-*traR* transformants (data not shown). This suggests that it is the combination of electroporation, antibiotic selection, and the growth-inhibitory effect of elevated TraR activity that prevents successful establishment of the introduced plasmid.

Effects of quorum sensing on growth rate have been reported for *R. leguminosarum* bv. viciae and *R. etli* CNPAF512 (7, 24, 58). Before its identification as an acyl-HSL, 3-OH-$C_{14:1}$-HSL produced by several biovars of *R. leguminosarum* was named small bacteriocin for its growth inhibitory effects (26). Subsequent studies demonstrated that small was an acyl-HSL synthesized via the activity of the LuxI-type protein CinI (37). CinI and its paired LuxR-type regulator CinR govern the complex hierarchy of multiple quorum-sensing systems in *R. leguminosarum*. The presence of the symbiotic plasmid pRL1JI partially suppresses production of 3-OH-$C_{14:1}$-HSL through inhibition of *cinI* expression, although this signal is still required for synthesis of plasmid-encoded acyl-HSLs. Addition of crude extracts or 3-OH-$C_{14:1}$-HSL to *R. leguminosarum* can arrest growth but does not cause a loss of viability (24). The mechanism of growth arrest requires genes carried on pRL1JI. *R. etli* CNPAF512 produces a saturated-long-chain (slc) acyl-HSL compound similar to 3-OH-$C_{14:1}$-HSL, referred to as 3-OH-(slc)-HSL, also via a protein called CinI (7). The *R. etli* CNPAF512 signal molecule inhibits growth of *R. leguminosa-*

rum strains sensitive to 3-OH-$C_{14:1}$-HSL. The *cinI* gene also influences *R. etli* CNPAF512 growth rate along with a second quorum-sensing system encoded by the *raiI* and *raiR* genes (56). *R. etli* CNPAF512 *raiR* and *cinI* single mutants and *cinI raiR* double mutants exhibit significantly longer lag times than the wild type upon inoculation and grow 1.5- to 2-fold more slowly, eventually reaching full density. These results suggest that 3-OH-(slc)-HSL is required for the normal growth rate of *R. etli*, in contrast to the case for *R. leguminosarum*. NGR234 has attributes of both systems; elevated expression of *traR* and addition of 3-oxo-C8-HSL lead to a reduction in growth rate, but rather than causing growth arrest, the growth rate is simply slowed.

Our findings indicate that the only genes on pNGR234a required for growth inhibition are *traR* and *traI*. Additionally, *A. tumefaciens* strains harboring pNGR234a are readily transformed with the *traR* expression plasmid, suggesting that pNGR234a is not sufficient for growth inhibition. A model in which growth inhibition is mediated by an interaction between TraR and functions encoded elsewhere in the NGR234 genome, perhaps including the genes responsible for synthesis of the pNGR234a-independent acyl-HSLs, is consistent with our findings (Fig. 6). We have isolated several NGR234 mutations that specifically suppress the growth-inhibitory effect of *traR* expression, none of which are located on pNGR234a (X. He and C. Fuqua, unpublished results).

TraR and TraM in other rhizobia. Marketon and González have identified genes encoding homologues of TraR and TraM with 80% identity to the pNGR234a gene products on the pRme41a plasmid from the alfalfa symbiont *S. meliloti* AK631 (42). In contrast to pNGR234a, the pRme41a plasmid from AK631 is not required for plant nodulation (27). Preliminary sequencing around the pRme41a *traR* and *traM* genes identified Mpf homologues of *trbH* and *trbI*, suggesting a gene organization similar to that of the Mpf cluster on pNGR234a (12). *S. meliloti* AK631 synthesizes a remarkable range of acyl-HSLs, and similar to our findings with NGR234, a portion of these are encoded by the pRme41a plasmid, while other long-chain acyl-HSLs are encoded within the remainder of the AK631 genome (42, 43). Mutation of *traR* significantly reduces synthesis of these acyl-HSLs in laboratory culture, suggesting that quorum sensing is active under these conditions (42). In contrast, our findings suggest that *traR* on pNGR234a is tightly controlled, keeping acyl-HSL levels low in laboratory cultures. Tight regulation of *traR* expression in NGR234 may be related to its role in growth rate control, as high-level activity would hamper growth. No effect of quorum sensing on growth rate has been reported for derivatives of *S. meliloti*.

Following submission of this work for publication, Wilkinson et al. published findings examining the regulation of conjugal transfer genes and growth inhibition in *R. leguminosarum* bv. viciae, which are in many ways consistent with our results with NGR234 (69). However, in *R. leguminosarum*, the *traR* homologue *triR* is activated by an adjacently encoded LuxR-type protein called BisR, which also inhibits 3-OH-$C_{14:1}$-HSL synthesis. There is no BisR homologue encoded adjacent to *traR* on the pNGR234a plasmid, but a second LuxR homologue, designated *y4qH*, is 250 kb away from the *tra-rep* cluster and could conceivably influence *traR* expression (12). Wilkinson et al. also found that the soluble translation elongation factor Ts (EF-Ts) is present at high levels in *R. leguminosarum* inhibited by 3-OH-$C_{14:1}$-HSL (69). It remains unclear whether this is a cause or an effect of growth inhibition. The EF-Ts levels have not been tested in growth-inhibited NGR234, but the protein is a possible target for TraR-dependent growth inhibition.

Complex quorum-sensing signaling pathways. Several different quorum-sensing microorganisms employ complex cascades with multiple acyl-HSLs and associated regulatory proteins. The best studied of these is *P. aeruginosa*, in which two quorum-sensing systems (Las and Rhl) function in a hierarchal, overlapping cascade that is hypothesized to provide staged expression of virulence factors for this pathogen in association with its hosts (5, 68). The rhizobia possess extremely complex quorum-sensing systems, and given the highly developed rhizobial-legume interactions, it is not surprising that quorum sensing contributes. Quorum sensing can influence different aspects of interaction with the host, ranging from modulatory to direct roles during nodulation (7, 37, 41, 56). We speculate that quorum sensing in NGR234 affects population dynamics in association with host plants. The growth regulation via TraR might function to control population expansion of NGR234 under crowded conditions, either within nodules or in the rhizospheres of nodulated plants.

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REFERENCES

1. **Akakura, R., and S. C. Winans.** 2002. Constitutive mutations of the OccR regulatory protein affect DNA bending in response to metabolites released from plant tumors. J. Biol. Chem. **277:**5866–5874.
2. **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-Microb. Interact. **11:**1119–1129.
3. **Charles, T. C., and T. M. Finan.** 1991. Analysis of a 1600-kilobase *Rhizobium meliloti* megaplasmid using defined deletions generated *in vivo*. Genetics **127:**5–20.
4. **Chen, C.-Y., and S. C. Winans.** 1991. Controlled expression of the transcriptional activator gene *virG* in *Agrobacterium tumefaciens* by using the *Escherichia coli lac* promoter. J. Bacteriol. **173:**1139–1144.
5. **Chugani, S. A., M. Whiteley, K. M. Lee, D. D'Argenio, C. Manoil, and E. P. Greenberg.** 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **98:**2752–2757.
6. **Cubo, M. T., A. Economou, G. Murphy, A. W. B. Johnston, and J. A. Downie.** 1992. Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. J. Bacteriol. **174:**4026–4035.
7. **Daniels, R., D. E. De Vos, J. Desair, G. Raedschelders, E. Luyten, V. Rosemeyer, C. Verreth, E. Schoeters, J. Vanderleyden, and J. Michiels.** 2002. Quorum-sensing in *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J. Biol. Chem. **277:**462–468.
8. **Ellis, J. G., A. Kerr, A. Petit, and J. Tempé.** 1982. Conjugal transfer of nopaline and agropine Ti-plasmids—the role of agrocinopines. Mol. Gen. Genet. **186:**269–273.
9. **Evans, K., L. Passador, R. Srikumar, E. Tsang, J. Nezezon, and K. Poole.** 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **180:**5443–5447.
10. **Farrand, S. K., I. Hwang, and D. M. Cook.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J. Bacteriol. **178:**4233–4247.
11. **Flores, M., P. Mavingui, L. Girard, X. Perret, W. J. Broughton, E. Martinez-Romero, G. Davila, and R. Palacios.** 1998. Three replicons of *Rhizobium* sp. strain NGR234 harbor symbiotic gene sequences. J. Bacteriol. **180:**6052–6053.

12. **Freiberg, C., R. Fellay, A. Balroch, W. J. Broughton, A. Rosenthal, and X. Perret.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394–401.
13. **Fuqua, C., M. Burbea, and S. C. Winans.** 1995. Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the *traM* gene. J. Bacteriol. **177:**1367–1373.
14. **Fuqua, C., and A. Eberhard.** 1999. Signal generation in autoinduction systems: synthesis of acylated homoserine lactones by LuxI-type proteins, p. 211–230. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. ASM Press, Washington, D.C.
15. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: acylhomoserine lactone signalling. Nat. Rev. Mol. Cell. Biol. **3:**685–695.
16. **Fuqua, C., M. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. **35:**439–468.
17. **Fuqua, C., and S. C. Winans.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. **178:**435–440.
18. **Fuqua, C., and S. C. Winans.** 1996. Localization of the OccR-activated and TraR-activated promoters that express two ABC-type permeases and the *traR* gene of the Ti plasmid pTiR10. Mol. Microbiol. **120:**1199–1210.
19. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI-type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. **176:**2796–2806.
20. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
21. **Gardener, B. B. M., and F. J. de Bruijn.** 1998. Detection and isolation of novel rhizopine-catabolizing bacteria from the environment. Appl. Environ. Microbiol. **64:**4944–4949.
22. **Genetello, C., N. Van Larebeke, M. Holsters, A. De Picker, M. Van Montagu, and J. Schell.** 1977. Ti plasmids of *Agrobacterium* as conjugative plasmids. Nature **265:**561–563.
23. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3076–3080.
24. **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.
25. **Hirsch, A. M., M. R. Lum, and J. A. Downie.** 2001. What makes the rhizobia-legume symbiosis so special? Plant Physiol. **127:**1484–1492.
26. **Hirsch, P. R.** 1979. Plasmid-determined bacteriocin production by *Rhizobium leguminosarum*. J. Gen. Microbiol. **113:**219–228.
27. **Huguet, T., C. Rosenberg, F. Casse-Delbart, P. de Lajudie, L. Jouanin, J. Batut, B. P., J.-S. Julliot, and J. Denarie.** 1983. Studies on *Rhizobium meliloti* plasmids and on their role in the control of nodule formation and nitrogen fixation: the pSym megaplasmids and the other large plasmids, p. 35–45. *In* A. Puhler (ed.), Molecular genetics of the bacteria-plant interaction. Springer-Verlag, New York, N.Y.
28. **Hwang, I., D. M. Cook, and S. K. Farrand.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J. Bacteriol. **177:**449–458.
29. **Hwang, I., P.-L. Li, L. Zhang, K. R. Piper, D. M. Cook, M. E. Tate, and S. K. Farrand.** 1994. TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. Proc. Natl. Acad. Sci. USA **91:**4639–4643.
30. **Hwang, I., A. J. Smyth, Z.-Q. Luo, and S. K. Farrand.** 1999. Modulating quorum sensing by antiactivation: TraM interacts with TraR to inhibit activation of Ti plasmid conjugal transfer genes. Mol. Microbiol. **34:**282–294.
31. **Kalogeraki, V. S., and S. C. Winans.** 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188:**69–75.
32. **Kaplan, H. B., and E. P. Greenberg.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163:**1210–1214.
33. **Kerr, A., P. Manigault, and J. Tempé.** 1977. Transfer of virulence in vivo and in vitro in *Agrobacterium*. Nature **265:**560–561.
34. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. I. Roop, 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.
35. **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*-tetradecanoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology **146:**2469–2480.
36. **Li, P.-L., and S. K. Farrand.** 2000. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. J. Bacteriol. **182:**179–188.
37. **Lithgow, J. K., A. Wilkinson, A. Hardman, B. Rodelas, F. Wisniewski-Dye, 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.
38. **Long, S. R.** 2001. Genes and signals in the rhizobium-legume symbiosis. Plant Physiol. **125:**69–72.
39. **Luo, Z.-Q., T. E. Clemente, and S. K. Farrand.** 2001. Construction of a derivative of *Agrobacterium tumefaciens* C58 that does not mutate to tetracycline resistance. Mol. Plant-Microbe Interact. **14:**98–103.
40. **Luo, Z.-Q., Y. Qin, and S. K. Farrand.** 2000. The antiactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. J. Biol. Chem. **275:**7713–7722.
41. **Marketon, M. M., S. A. Glenn, A. Eberhard, and J. E. González.** 2002. Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. J. Bacteriol. **185:**5686–5695.
42. **Marketon, M. M., and J. E. González.** 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J. Bacteriol. **184:**3466–3475.
43. **Marketon, M. M., M. R. Gronquist, A. Eberhard, and J. E. González.** 2002. Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones. J. Bacteriol. **184:**5686–5695.
44. **Mavingui, P., M. Flores, X. Guo, G. Davila, X. Perret, W. J. Broughton, and R. Palacios.** 2002. Dynamics of genome architecture in *Rhizobium* sp. strain NGR234. J. Bacteriol. **184:**171–176.
45. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. 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.
46. **Mersereau, M., G. J. Pazour, and A. Das.** 1990. Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene **90:**149–151.
47. **Morrison, N. A., C. Y. Hau, M. J. Trinick, J. Shine, and B. G. Rolfe.** 1983. Heat curing of a Sym plasmid in a fast-growing *Rhizobium* sp. that is able to nodulate legumes and the nonlegume *Parasponia* sp. J. Bacteriol. **153:**527–531.
48. **Murphy, P. J., N. Heycke, Z. Banfalvi, M. E. Tate, F. J. de Bruijn, A. Kondorosi, J. Tempe, and J. Schell.** 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc. Natl. Acad. Sci. USA **84:**493–497.
49. **Parsek, M. R., and E. P. Greenberg.** 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA **97:**8789–8793.
50. **Perret, X., C. Staehelin, and W. J. Broughton.** 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:**180–201.
51. **Piper, K. R., S. Beck von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448–450.
52. **Piper, K. R., S. Beck von Bodman, I. Hwang, and S. K. Farrand.** 1999. Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. Mol. Microbiol. **32:**1077–1089.
53. **Piper, K. R., and S. K. Farrand.** 2000. Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. J. Bacteriol. **182:**1080–1088.
54. **Pueppke, S. G., and W. J. Broughton.** 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. Mol. Plant-Microbe Interact. **12:**293–318.
55. **Qin, Y., Z.-Q. Luo, A. J. Smyth, P. Gao, S. Beck von Bodman, and S. K. Farrand.** 2000. Quorum-sensing signal binding results in dimerization of TraR and its release from membranes into the cytoplasm. EMBO J. **19:**5212–5221.
56. **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 of *Phaseolus vulgaris*. J. Bacteriol. **180:**815–821.
57. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
58. **Schripsema, J., K. E. E. de Rudder, T. B. van Vliet, P. P. Lankhorst, E. de Vroom, J. W. Kijne, and A. A. N. van Brussel.** 1996. Bacteriocin *small* of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum-sensing cotranscription factors. J. Bacteriol. **178:**366–371.
59. **Shaw, P., G. Ping, S. L. Daly, C. Cha, J. E. J. Cronan, 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.
60. **Stevens, A. M., and E. P. Greenberg.** 1999. Transcriptional activation by LuxR, p. 231–242. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. ASM Press, Washington, D.C.

61. **Summers, W. C.** 1970. A simple method for extraction of RNA from E. coli utilizing diethyl pyrocarbonate. Anal. Biochem. **33:**459–463.
62. **Swiderska, A., A. K. Berndtson, M.-R. Cha, L. Li, G. M. J. Beaudoin III, J. Zhu, and C. Fuqua.** 2001. Inhibition of the *Agrobacterium tumefaciens* TraR quorum-sensing regulator: interactions with the TraM anti-activator. J. Biol. Chem. **276:**49449–49458.
63. **Tempé, J., A. Petit, Holsters, M. Van Montagu, and J. Schell.** 1977. Thermosensitive step associated with transfer of the Ti plasmid during conjugation: possible relation to transformation in crown gall. Proc. Natl. Acad. Sci. USA **74:**2848–2849.
64. **Trinick, M. J.** 1980. Relationships among the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora*. J. Appl. Bacteriol. **49:**39–53.
65. **Viprey, V., A. Rosenthal, W. J. Broughton, and X. Perret.** 2000. Genetic snapshots of the *Rhizobium* species NGR234 genome. Genome Biol. **1:**14.1–14.7.
66. **Welch, M., D. E. Todd, N. A. Whitehead, S. J. McGowan, B. W. Bycroft, and G. P. C. Salmond.** 2000. *N*-Acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. EMBO J. **19:**631–641.
67. **Wexler, M., D. Gordon, and P. J. Murphy.** 1995. The distribution of inositol rhizopine genes in *Rhizobium* populations. Soil Biol. Biochem. **27:**531–537.
68. **Whitehead, N. A., A. M. L. Barnard, H. Slater, N. J. L. Simpson, and G. P. C. Salmond.** 2001. Quorum-sensing in gram-negative bacteria. FEMS Microbiol. Rev. **25:**365–404.
69. **Wilkinson, A., V. Danino, F. Wisniewski-Dyé, J. K. Lithgow, and J. A. Downie.** 2002. *N*-Acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. J. Bacteriol. **184:**4510–4519.
70. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Res. **18:**6531–6535.
71. **Winans, S. C., J. Zhu, and M. I. Moré.** 1999. Cell density-dependent gene expression by *Agrobacterium tumefaciens* during colonization of crown gall tumors, p. 117–128. *In* G. M. Dunny and S. C. Winans (ed.), Cell-cell signaling in bacteria. ASM Press, Washington, D.C.
72. **Woodcock, D. M., C. P. J., J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. **17:**3469–3478.
73. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. Nature **362:**446–448.
74. **Zhu, J., J. W. Beaber, M. I. Moré, C. Fuqua, A. Eberhard, and S. C. Winans.** 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol. **180:**5398–5405.
75. **Zhu, J., and S. C. Winans.** 1999. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. Proc. Natl. Acad. Sci. USA **96:**4832–4837.