

# The *cin* and *rai* Quorum-Sensing Regulatory Systems in *Rhizobium leguminosarum* Are Coordinated by ExpR and CinS, a Small Regulatory Protein Coexpressed with CinI<sup>∇</sup>

Anne Edwards,<sup>1</sup> Marijke Frederix,<sup>1</sup> Florence Wisniewski-Dyé,<sup>2</sup> Jacob Jones,<sup>1</sup>  
Angeles Zorreguieta,<sup>3</sup> and J. Allan Downie<sup>1\*</sup>

John Innes Centre, Colney, Norwich NR4 7UH, United Kingdom<sup>1</sup>; Université Lyon 1, CNRS UMR 5557 Ecologie Microbienne, 69622 Villeurbanne, France<sup>2</sup>; and Fundación Instituto Leloir, IIBBA-CONICET, FCEyN, University of Buenos Aires, Patricias Argentinas 435, Buenos Aires, Argentina<sup>3</sup>

Received 20 November 2008/Accepted 23 February 2009

To understand how the *Rhizobium leguminosarum* *rai*-*raiR* quorum-sensing system is regulated, we identified mutants with decreased levels of RaiI-made *N*-acyl homoserine lactones (AHLs). A LuxR-type regulator, ExpR, is required for *raiR* expression, and RaiR is required to induce *raiI*. Since *raiR* (and *raiI*) expression is also reduced in *cinI* and *cinR* quorum-sensing mutants, we thought CinI-made AHLs may activate ExpR to induce *raiR*. However, added CinI-made AHLs did not induce *raiR* expression in a *cinI* mutant. The reduced *raiR* expression in *cinI* and *cinR* mutants was due to lack of expression of *cinS* immediately downstream of *cinI*. *cinS* encodes a 67-residue protein, translationally coupled to CinI, and *cinS* acts downstream of *expR* for *raiR* induction. Cloned *cinS* in *R. leguminosarum* caused an unusual collapse of colony structure, and this was delayed by mutation of *expR*. The phenotype looked like a loss of exopolysaccharide (EPS) integrity; mutations in *cinI*, *cinR*, *cinS*, and *expR* all reduced expression of *plyB*, encoding an EPS glycanase, and mutation of *plyB* abolished the effect of cloned *cinS* on colony morphology. We conclude that CinS and ExpR act to increase PlyB levels, thereby influencing the bacterial surface. CinS is conserved in other rhizobia, including *Rhizobium etli*; the previously observed effect of *cinI* and *cinR* mutations decreasing swarming in that strain is primarily due to a lack of CinS rather than a lack of CinI-made AHL. We conclude that CinS mediates quorum-sensing regulation because it is coregulated with an AHL synthase and demonstrate that its regulatory effects can occur in the absence of AHLs.

Production of *N*-acyl homoserine lactones (AHLs) is common to many plant-associated bacteria (7), in which it is usually associated with population density-dependent regulation of genes affecting adaptive responses (49). Within the family *Rhizobiaceae*, population density-regulated gene expression (quorum sensing) mediated via AHLs has been identified in several agrobacteria and rhizobia (13, 51). In *Agrobacterium* spp., quorum-sensing regulation was initially identified as a mechanism of regulating plasmid transfer. As the bacterial population density increases, plasmid transfer genes are induced by TraR in response to AHLs made by TraI (55). In several rhizobia, *traI*-like AHL synthase genes are also in an operon along with plasmid transfer genes (13).

There are other quorum-sensing loci in different strains of rhizobia. In *Sinorhizobium meliloti* strain Rm1021, AHLs produced by SinI activate SinR and ExpR, LuxR-type regulators, to induce several genes, including those determining the production of an exopolysaccharide, exopolysaccharide II (EPS-II) (17, 23, 24, 35), that plays an important role in the symbiosis. In *S. meliloti*, two LuxR-type regulators, VisN and VisR, are involved in chemotaxis and motility (24, 44).

*Rhizobium etli* has multiple AHL synthase genes (9, 39), but the functions of many of the regulated genes remain to be established. The *cinR* and *cinI* genes are required for normal symbiotic nitrogen fixation and swarming in *R. etli* (5, 9, 11) and for normal levels of expression of *raiI*, which encodes another AHL synthase. The expression of *raiI* in *R. etli* is regulated by RaiR (39).

Analysis of AHLs produced by strain A34 of *Rhizobium leguminosarum* bv. *viciae* led to the characterization of four LuxI-type AHL synthases (RhiI, CinI, RaiI, and TraI) and five LuxR-type regulators (RhiR, CinR, RaiR, TraR, and BisR) (8, 31, 50, 53). In this strain, the *cinI* and *cinR* genes are chromosomally located; CinI produces *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (3-OH-C<sub>14:1</sub>-HSL) (20, 31), CinR induces *cinI* expression in response to this AHL (31), and this appears to be associated with adaptation to starvation and salt stress (47). Mutation of *cinI* or *cinR* affects the expression of the other three AHL synthase genes in *R. leguminosarum* bv. *viciae* strain A34. Thus, in a *cinI* mutant, the expression of *raiI* is reduced, resulting in very low levels of 3-OH-C<sub>8</sub>-HSL, the major AHL made by RaiI (53). Similarly, the expression levels of the *traI* and *rhiI* genes on the symbiotic plasmid pRL1JI are reduced in *cinI* and *cinR* mutants (31). RhiI-made AHLs activate RhiR to induce the expression of the *rhiABC* operon in *R. leguminosarum* bv. *viciae* (38), enhancing the interaction with the legume host (8).

\* Corresponding author. Mailing address: John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7 UH, United Kingdom. Phone: 44-1603-450207. Fax: 44-1603-450045. E-mail: allan.downie@bbsrc.ac.uk.

<sup>∇</sup> Published ahead of print on 6 March 2009.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source
<i>R. leguminosarum</i> strains		
8400	Strain lacking a symbiotic plasmid	29
8401	Str <sup>r</sup> derivative of strain 8400	29
A34	Strain 8401 containing pRL1JI (pSym)	14
A616	8401 <i>prsD1</i> ::Tn5	16
A552	8401 <i>cinR1</i> ::Tn5	31
A702	8401 <i>expR1</i> ::Tn5-gus	This work
A740	8401 <i>cinI3</i> ::Spc <sup>r</sup>	31
A789	8401 <i>rail7</i> ::Tn5	53
A797	8401 <i>cinI3</i> ::Spc <sup>r</sup> <i>rail7</i> ::Tn5	53
A802	8401 <i>railR8</i> ::Tn5	53
A1085	8400 <i>expR1</i> ::Tn5-gus	This work
A1102	8401 carrying <i>cinS</i> ::Spc <sup>r</sup>	This work
<i>R. etli</i> CNPAF512	WT <i>R. etli</i>	10
<i>R. etli</i> FAJ4006	CNPAF512 <i>cinI</i> ::Tn5-gus	10
<i>A. tumefaciens</i> NT1/pZLR4	<i>traG-lacZ</i> -based AHL detection strain	7
<i>C. violaceum</i> CV026	AHL detection strain	33
Plasmids		
pIJ7516	Cosmid carrying <i>cinRI</i> locus	31
pIJ7910	<i>cinI'</i> - <i>lacZ</i> in pMP220	31
pIJ9001	Cosmid carrying <i>railR</i> region	53
pIJ9115	7-kb EcoRI fragment from strain A702 carrying part of the Tn5-gus in pBluescript SK	This work
pIJ9123	Cosmid carrying <i>expR</i> region in pLAFR1	This work
pIJ9229	<i>expR</i> on 3-kb EcoRI fragment from pIJ9123 in pBluescript KS	This work
pIJ9252	<i>plyB'</i> - <i>lacZ</i> fusion in pMP220	This work
pIJ9263	<i>expR'</i> - <i>lacZ</i> fusion in pMP220	This work
pIJ9272	<i>railR'</i> - <i>lacZ</i> fusion in pMP220	53
pIJ9276	<i>railR</i> in pBBR1-MCS5	53
pIJ9280	<i>rail'</i> - <i>lacZ</i> fusion in pMP220	53
pIJ9493	<i>expR</i> in pBBR1-MCS5	This work
pIJ9655	<i>cinI</i> and truncated <i>cinS</i> in pKT230	This work
pIJ9692	<i>cinS</i> in pKT230	This work
pIJ9769	<i>expR</i> in pBBR1-MCS2	This work
pMP220	Broad-host-range <i>lacZ</i> expression vector; Tc <sup>r</sup>	45
pKT230	Broad-host-range <i>lacZ</i> expression vector; Kan <sup>r</sup>	2
pBBR1-MCS2	Broad-host-range plasmid; Kan <sup>r</sup>	27
pBBR1-MCS5	Broad-host-range plasmid; Gent <sup>r</sup>	27

The *cinI* and *cinR* quorum-sensing genes control induction of the *traI* and *traR* quorum-sensing regulons via CinI-made 3-OH-C<sub>14:1</sub>-HSL, which activates BisR (another LuxR-type regulator) to induce *traR* and hence *traI* (12). However, the mechanism by which *cinI* and/or *cinR* control *rail* and *railR* expression has not been established. In this work we demonstrate that *rail* and *railR* expression requires both *expR* and a small gene (*cinS*) cotranscribed with *cinI*. CinS also regulates the expression of *plyB* encoding an extracellular glycanase and is required for swarming of *R. etli*.

#### MATERIALS AND METHODS

**Microbiological techniques.** Rhizobia were grown at 28°C in TY medium (4) or Y minimal medium (43) containing mannitol (0.2% [wt/vol]) (Y-mannitol medium) as the carbon source. Antibiotics were added as appropriate to maintain selection for plasmids. Bacterial growth and  $\beta$ -galactosidase activity were measured using a Perkin-Elmer MBA2000 spectrophotometer as described previously (53) using at least six independent cultures. To measure formation of biofilm rings, bacteria were inoculated into 100 ml of Y-mannitol medium in 250-ml Erlenmeyer flasks using 2 ml of TY cultures grown to an optical density at 600 nm (OD<sub>600</sub>) of 1.0. Flasks were shaken at 300 rpm at 28°C, and growth of biofilm rings was assayed after 5 days. The biofilm rings were harvested using cotton buds and resuspended in 1 ml H<sub>2</sub>O, and crystal violet was added to a final concentration of 0.01% from a fresh 0.4% (wt/vol) aqueous stock and left for 30

min. The bacteria were washed four times with water and then resuspended in 1 ml ethanol. After 1 h, the samples were centrifuged, and the OD<sub>575</sub> was determined. Colony morphology changes were assayed by inoculating TY plates directly from glycerol stocks and incubating the plates for 3 days at 28°C. Swarming of *R. etli* was assayed in yeast extract-mannitol (YEM) swarm plates containing 0.7% agar as described previously (10) after growth at 28°C for 4 days. Nodulation tests were done using peas (*Pisum sativum* L.) variety Frisson as described previously (25), using a minimum of 16 matched plants per test.

**Bacterial strains.** The strains and plasmids used are listed in Table 1 or in the text. *R. leguminosarum* strain 8400 lacks a symbiotic plasmid, and all *R. leguminosarum* strains were derived from strain 8400 or its streptomycin-resistant derivative, strain 8401. A34 is a derivative of strain 8401 carrying the symbiotic plasmid pRL1JI. Plasmids were mobilized into *Rhizobium* by triparental matings using a helper plasmid. Strain 8401 was mutagenized with Tn5-gus by using *Escherichia coli* strain MM294/pRK600::Tn5-gus as a donor of the suicide plasmid pRK600::Tn5-gus essentially as described previously (41). A population of about 8,000 colonies was screened for impaired AHL production by picking colonies onto a lawn of the AHL biosensor strain *Chromobacterium violaceum* CV026 (33) to identify mutants that did not induce the purple pigment violacein. One of these mutants (A702; *expR*) was severely affected for production of RaiI-made AHLs, and small-bacteriocin tests revealed that strain A702 retained the ability to produce 3-OH-C<sub>14:1</sub>-HSL. Strain A1085 was made by plating bacteriophage RL38 (6) on strain A702 and using the purple lysate to transduce strain 8400, selecting for kanamycin resistance. To make strain A1102 (*cinS*::Spc<sup>r</sup>), *cinS* with 1-kb flanking regions was first amplified by PCR using primers CTGAAGAGCGGCGGCTTCAAGCTC and GAGAACTTACGGGC CGCTATTGATTTC containing introduced NotI sites (bold face). The product





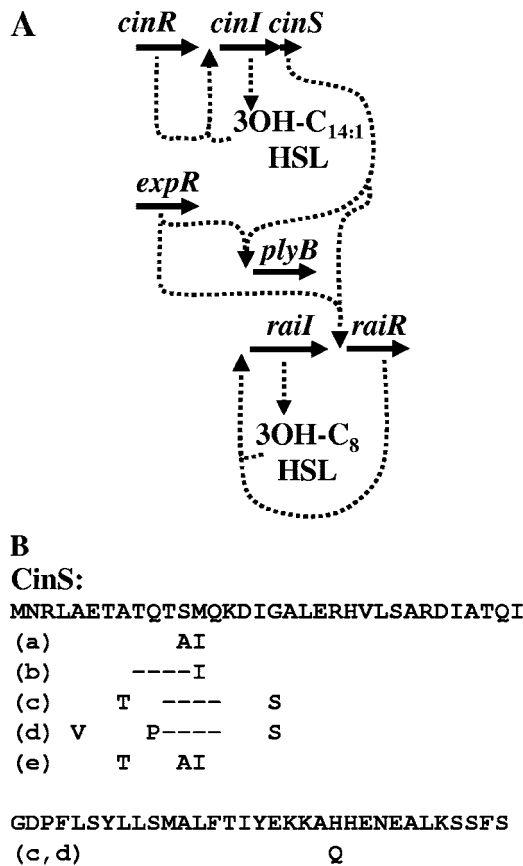


FIG. 2. (A) Model of quorum-sensing regulation in *R. leguminosarum* strain 8401. The *cinS* gene is cotranscribed with *cinI* and is predicted to be translationally coupled to *cinI* and so is induced by CinR in response to CinI-made AHL. Both *cinS* and *expR* are required for full induction of *raiR* and *plyB*; RaiR regulates *raiI* in response to RaiI-made AHL. (B) Predicted sequence of CinS from strain 8401 (top line) and the differences in the conserved homologues in *R. leguminosarum* bv. *viciae* 3841 (GenBank nucleotide accession no. AM236080) and *R. leguminosarum* bv. *trifolii* strain WSM1325 (GenBank protein accession no. EDR73518) (line a), *R. leguminosarum* bv. *trifolii* strain WSM2304 (GenBank protein accession no. ACI55940) (line b), *R. etli* strains CNPAF512 (GenBank nucleotide accession no. AF393621) and CIAT652 (GenBank protein accession no. ACE92023) (line c), *R. etli* strain CFN42 (GenBank nucleotide accession no. CP000133) (line d), and *Mesorhizobium tianshanense* (GenBank nucleotide accession no. DQ123807) (line e). The residues different from those in CinS in strain 8401 are shown below the sequence; deletions are shown by dashes.

strain A802 (Fig. 1A, lane 8). Conversely, *raiR* cloned behind a vector promoter on pIJ9276 (Fig. 1A, lane 6) restored AHL production to the *expR* mutant A702 (as well as to the *raiR* mutant; Fig. 1A, lane 7), suggesting that ExpR induces *raiR* and hence *raiI* (Fig. 2).

***raiR* (and hence *raiI*) expression is regulated by both ExpR and a novel small protein, CinS.** The *expR* mutation in strain A702 strongly reduced expression of both *raiI'*-*lacZ* (on pIJ9280) and *raiR'*-*lacZ* (on pIJ9272) (Table 2). These assays were done in Y medium, in which *raiI* and *raiR* expression is significantly higher than that seen previously (53) in TY medium. Mutation of *cinI* (Table 2) and *cinR* (data not shown) similarly reduced *raiI* and *raiR* expression. Mutation of *raiR* or *raiI* did not affect *raiR* expression, although as expected (53),

they did reduce *raiI* expression (Table 2). Cloned *raiR* (pIJ9276) caused high levels of *raiI* expression in the *expR* mutant; A702/pIJ9280 (*raiI'*-*lacZ*) carrying pIJ9276 (constitutive *raiR*) had 15,286 ( $\pm 300$ ) units of  $\beta$ -galactosidase activity, similar to the high levels of *raiI'*-*lacZ* expression seen previously with cloned *raiR* in the *cinI* and *cinR* mutants or in strain 8401 (53).

Induction of *raiR* requires *expR*, *cinI*, and *cinR*, but there is no known AHL synthase associated with ExpR. To test whether ExpR induced *raiR* in response to CinI-made 3-OH-C<sub>14:1</sub>-HSL, we added 1  $\mu$ M 3-OH-C<sub>14:1</sub>-HSL to the growth medium of the *cinI* mutant A740 carrying *raiR'*-*lacZ* (pIJ9272) but saw no induction ( $236 \pm 37$  units); we could not restore *raiI* or *raiR* expression in the *cinI* mutant with other AHLs, including C<sub>6</sub>-HSL, 3-O-C<sub>8</sub>-HSL, and crude AHL extracts from strain 8401 (data not shown). This suggested that the effect of the *cinI* mutation may be due to polarity. Although there is only a short gap between the coding region of *cinI* and the adjacent convergently transcribed gene (31), there is a short open reading frame encoding a predicted protein of 67 residues that appears to be translationally coupled to CinI; as described below, this is indeed a gene which we named *cinS* (Fig. 2).

A *cinS* mutant (strain A1102) produced normal levels of CinI-made 3-OH-C<sub>14:1</sub>-HSL (data not shown), and A1102/pIJ9272 (*cinI'*-*lacZ*) had normal levels of  $\beta$ -galactosidase expression ( $20,628 \pm 2,694$  units compared with  $24,692 \pm 1,940$  units seen with the control 8401/pIJ9272). This contrasts with the relatively low expression ( $986 \pm 75$  units) in the *cinI* mutant A740 carrying pIJ9272. Strain A1102 (*cinS*) lacked RaiI-made AHLs (Fig. 1B, lane 2); like the *expR* mutant (and *cinI* and *cinR* mutants), it did not induce *raiI'*-*lacZ*, apparently due to a lack of *raiR* expression (Table 2). Cloned *cinS* behind a vector promoter (pIJ9692) restored the expression of *raiR'*-*lacZ* in the *cinI*, *cinR*, and *cinS* mutants (Fig. 3A) and restored formation of the RaiI-made AHLs in the *cinS* and *cinI* mutants A1102 and A740 (Fig. 1B, lane 3, and Fig. 1C, lane 3), and in the *cinR* mutant A552 (data not shown). In contrast, pIJ9655 carrying cloned *cinI* and part of *cinS* (encoding the N-terminal 36 residues) did not complement *raiR'*-*lacZ* expression in the *cinI* mutant (A740/pIJ9272 had  $278 \pm 23$  units compared with over 1,000 units in the WT; Table 2); pIJ9655 (*cinI*) also did not restore formation of RaiI-made AHLs to the *cinI* mutant, although as expected, it restored production of CinI-made AHLs (data not shown). The complementation of *raiR* expres-

TABLE 2. Effect of an *expR* mutation on *raiR'*-*lacZ* and *raiI'*-*lacZ* expression<sup>a</sup>

Strain (relevant genotype)	Expression <sup>b</sup> of:	
	<i>raiI'</i> - <i>lacZ</i> (pIJ9280)	<i>raiR'</i> - <i>lacZ</i> (pIJ9272)
8401 (WT)	5,873 $\pm$ 10	1,075 $\pm$ 146
A702 ( <i>expR</i> )	340 $\pm$ 25	282 $\pm$ 44
A740 ( <i>cinI</i> )	346 $\pm$ 18	302 $\pm$ 53
A1102 ( <i>cinS</i> )	362 $\pm$ 9	318 $\pm$ 30
A789 ( <i>raiI</i> )	462 $\pm$ 16	1,135 $\pm$ 99
A802 ( <i>raiR</i> )	344 $\pm$ 13	1,030 $\pm$ 7

<sup>a</sup> Bacteria were cultured for 48 h in Y-mannitol medium.

<sup>b</sup> Data shown are mean values for  $\beta$ -galactosidase activity (in Miller units)  $\pm$  standard errors, based on averages of at least six assays.

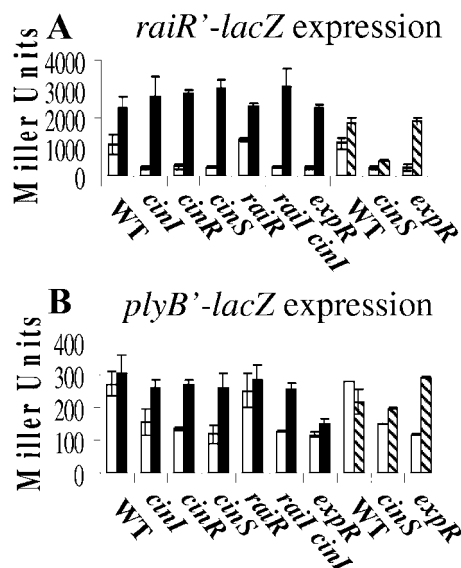


FIG. 3. Effects of cloned *cinS* and *expR* on *raiR'*-*lacZ* and *plyB'*-*lacZ* expression in quorum-sensing mutants of *R. leguminosarum*. Strains were grown in Y-mannitol medium, and  $\beta$ -galactosidase activity was assayed after 48 h (for *raiR'*-*lacZ*) or 72 h (for *plyB'*-*lacZ*) of growth. The WT strain (strain 8401) and mutant strains were used. The mutant strains used were *cinI* (strain A740), *cinR* (A552), *cinS* (A1102), and *raiI* (A789) single mutants, the *raiI cinI* double mutant (A797), and *expR* mutant (A1085) (in this experiment A1085 was used instead of A702 to facilitate plasmid selection). (A) *raiR'*-*lacZ* activities of WT and mutants with cloned *cinS* on pIJ9692 (black bars) or cloned *expR* on pIJ9769 (hatched bars) or the strains containing the appropriate vector controls pK230 or pBBR1-MCS2 (white bars). (B) *plyB'*-*lacZ* activities of WT and mutants with cloned *cinS* on pIJ9692 (black bars) or cloned *expR* on pIJ9769 (hatched bars) or the strains containing the appropriate vector control, pK230 or pBBR1-MCS2 (white bars).

sion in the *cinI* mutant by *cinS*, but not by *cinI*, confirms the prediction that the *cinI* mutation in A740 is polar on *cinS* and that *cinI* and *cinS* are cotranscribed (Fig. 2A).

Taken together, these observations show that the lack of RaiI-made AHLs in *cinI* and *cinR* mutants is due to a lack of *cinS* expression and that CinI-made AHLs are not essential for *raiI* expression if *cinS* is expressed constitutively. The effect of CinS is on *raiR* expression, because cloned *cinS* on pIJ9692 restored *raiR'*-*lacZ* expression to normal levels in the *cinI* (A740) and *cinR* (A552) mutants (Fig. 3A). Thus, both CinS and ExpR are normally required for *raiR* (and hence *raiI*) induction (Fig. 2), and CinI-made AHL is not required if *cinS* is expressed constitutively. Cloned *cinS* also restored *raiR* expression in a *raiI cinI* double mutant, strain A797 (Fig. 3A), which makes no detectable AHLs, confirming that CinS can act independently of AHLs. Cloned *cinS* (pIJ9692) did not restore production of RaiI-made AHLs (data not shown) or *raiI'*-*lacZ* expression in the *raiR* mutant A802; *raiI'*-*lacZ* expression on pIJ9280 in the *raiR* mutant A802 was low ( $75 \pm 9$  units compared with  $4,089 \pm 347$  units in WT strain 8401) and was not affected by cloned *cinS* ( $79 \pm 18$  units), showing that RaiI acts downstream of CinS for induction of *raiI*.

Cloned *cinS* (pIJ9692) restored both *raiR* expression (Fig. 3A) and the production of RaiI-made AHLs to the *expR* mutant A702 (Fig. 1C, lane 5), but in the *cinS* mutant A1102,

cloned *expR* (pIJ9769) only very slightly increased both AHL production (Fig. 1B, lane 4) and *raiR'*-*lacZ* (pIJ9272) expression (Fig. 3A). This shows that CinS acts downstream of ExpR (the *expR* gene on pIJ9769 is functional because it complemented the *expR* mutant; Fig. 3A).

These data are not consistent with a model in which ExpR directly regulates *raiR* or *raiI* expression. The observations point toward both CinS and ExpR having an effect on the levels of *raiR* transcript (Fig. 2A), although high-level expression of CinS can compensate for the absence of ExpR.

***expR* expression and *cinIS* expression are not interdependent.** The *expR* mutant A702 carrying *cinI'*-*lacZ* (pIJ7910) expressed *cinI'*-*lacZ* normally ( $23,114 \pm 1,753$  units compared with  $24,692 \pm 1,940$  units in strain 8401/pIJ7910) and produced normal levels of CinI-made 3-OH-C<sub>14:1</sub>-HSL (data not shown), showing that *expR* is not required for expression of the *cinIS* operon. These observations are different from those made in *S. meliloti*, in which *expR* and SinI-made AHLs are required for full induction of *sinI* (23, 34).

We tested whether *expR* expression is regulated by CinR or by CinI-made AHLs. An *expR'*-*lacZ* fusion (pIJ9263) was expressed similarly in the control strain 8401 and the *cinR* mutant A552 ( $856 \pm 45$  units compared with  $902 \pm 23$  units, respectively). Therefore, *expR* expression is not regulated by CinR. Similar levels of *expR'*-*lacZ* expression were seen with pIJ9263 in strain A740 (*cinI*) ( $911 \pm 56$  units), A789 (*raiI*) ( $813 \pm 119$  units), A797 (*raiI cinI*) ( $865 \pm 86$  units), and A802 (*raiR*) ( $732 \pm 164$  units), showing that *expR* expression is independent of the Cin and Rai AHL-based regulatory systems.

***cinS* influences colony morphology and formation of biofilm rings.** Mutation of *cinS*, *cinR*, *cinI*, or *expR* had no obvious effect on the growth rate of *R. leguminosarum* in minimal or complex media or on the symbiosis (nodulation and nitrogen fixation) of peas (data not shown), although the colonies on TY medium appeared slightly more mucoid than normal. Plated cultures of strain 8401 (WT) carrying cloned *cinS* (on pIJ9692) had an unusual phenotype, because the primary inoculum on TY plates appeared to age prematurely. Normally the primary inoculum region is quite mucoid after about 2 days of growth but appears to “collapse” or dry up after about 7 days of incubation. However, cloned *cinS* (pIJ9692) caused this collapse to occur in the control, strain 8401, after about 3 days (Fig. 4A). This effect of cloned *cinS* was independent of AHL-dependent quorum-sensing regulation, because the collapse phenotype was induced by pIJ9692 in the *cinR* mutant (Fig. 4B), and similar results (data not shown) were seen with the *cinI*, *cinS*, *raiR*, and *raiI* mutants as well as the *raiI cinI* double mutant. However, the *expR* mutation in strain A702 blocked the *cinS*-induced collapse at 3 days (Fig. 4C). This suggests that, like *raiR* induction, both *expR* and *cinS* are required to induce the colony collapse, but since the effect occurred in the *raiI* and *raiR* mutants, the collapse cannot be induced via an effect of ExpR and CinS on the *raiI*-*raiR* regulatory system.

Most colony mucoidicity is due to the acidic exopolysaccharide (EPS), and so we postulated that the collapse phenotype might be caused by premature degradation of the EPS. PlyA and PlyB are extracellular glycanases that can cleave the acidic EPS (16, 56) and so we tested whether they were quorum sensing regulated. A *plyB'*-*lacZ* reporter fusion (on pIJ9252) was induced during stationary phase (Fig. 5), and its expression

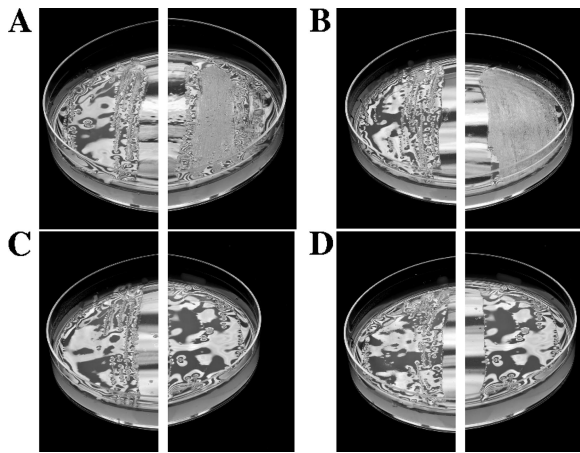


FIG. 4. Cloned *cinS* induced a colony collapse phenotype. The photographs show cultures of strains grown for 3 days on TY agar medium. For each strain, the photograph on the left shows the strain grown with the vector (pKT230) control and the photograph on the right shows the strain with *cinS* on pIJ9692. Strains 8401 (WT) (A), A552 (*cinR*) (B), A702 (*expR*) (C), and A616 (*plyB*) (D) are shown. After 2 days, the appearance of *R. leguminosarum* 8401/pIJ9692 and A552/pIJ9692 cultures looked normal (not shown) but collapsed, forming a dry appearance after 3 days. Cloned *cinS* had no effect on strain A616 (*plyB*) even after prolonged culture, but with strain A702 (*expR*), a delayed colony collapse was observed (not shown). pIJ9692 (*cinS*) induced a similar collapse to that seen in panels A and B in strains A740 (*cinI*), A1102 (*cinS*), A789 (*raiI*), A802 (*raiR*), and A797 (*raiI cinI*) (not shown).

was significantly decreased by mutations in *cinI*, *expR*, *cinS*, and *cinR* (Fig. 5). No change was seen with the *plyA'-lacZ* fusion (data not shown) in those mutants.

As with *raiR'-lacZ*, *plyB'-lacZ* expression in the *cinR*, *cinI*, and *cinS* mutants was restored by cloned *cinS* (Fig. 3B); its expression was also restored by cloned *expR* in the *expR*, but not the *cinS*, mutant (Fig. 3B). The correlation of *plyB* and *raiR* expression raised the possibility that *plyB* may be regulated by RaiR, but the expression of *plyB'-lacZ* in the *raiR* mutant showed that was not the case (Fig. 3B). Mutation of *expR* reduced *plyB'-lacZ* expression, but in this case cloned *cinS* did not restore *plyB* expression (Fig. 3B); this correlates with the

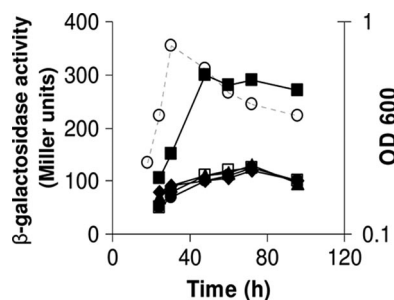


FIG. 5. Decrease of *plyB-lacZ* expression in *cinI*, *cinR*, *cinS*, and *expR* mutants. Expression of *plyB* was assayed throughout growth in Y-mannitol medium using pIJ9252 (*plyB'-lacZ*) in the WT strain 8401 (solid squares), the *cinI* mutant A740 (solid triangles), the *cinR* mutant A552 (solid diamonds), the *cinS* mutant A1102 (open squares), and the *expR* mutant A702 (solid circles). The growth of strain 8401 measured at OD<sub>600</sub> is shown as open circles and was very similar for all strains.

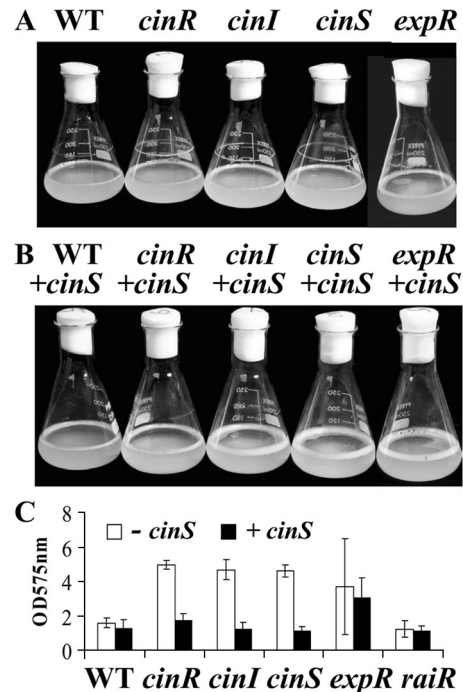


FIG. 6. Effects of cloned *cinS* on biofilm rings produced by quorum-sensing mutants. (A) Biofilm rings formed at the air-liquid interface of shaking flask cultures of strains grown for 5 days in Y-mannitol medium are shown. Strains A552 (*cinR*), A740 (*cinI*), A1102 (*cinS*), and A702 (*expR*) produced enhanced biofilm rings compared to that seen with 8401 (WT), although the biofilm formed by strain A702 was less stable and tended to be shed into the growth medium. (B) Cloned *cinS* (on pIJ9692) suppressed the formation of the enhanced biofilm rings in the *cin* mutants. (C) Biofilm rings similar to those illustrated above were quantified by staining with crystal violet following growth of strain 8401 (WT), A552 (*cinR*), A740 (*cinI*), A1102 (*cinS*), A702 (*expR*), and A802 (*raiR*) carrying the pKT230 vector (absence of *cinS* [- *cinS*]) (open bars) or *cinS* on pIJ9692 (*cinS* present [+ *cinS*]) (black bars). Standard deviations ( $n = 3$ ) are indicated by the error bars.

observation (Fig. 4C) that the *expR* mutation delayed the colony collapse phenotype induced by cloned *cinS*. These observations are consistent with both ExpR and CinS being required for full *plyB* expression, but increased expression of *cinS* can cause an enhanced amount of PlyB, which causes degradation of EPS and a resulting collapse in colony structure. Consistent with this, cloned *cinS* did not induce the colony collapse in the *plyB* mutant A616 (Fig. 4D).

After 3 to 4 days in shaken flask cultures in Y-mannitol medium, the *cinS* mutant produced a biofilm ring that was enhanced compared with that of the control strain 8401 (Fig. 6A), and this was restored to normal by cloned *cinS* on pIJ9692 (Fig. 6B). Similar results were seen with the *cinI* and *cinR* mutants (Fig. 6). Biofilm rings produced by strain 8401 and the *cinS*, *cinI*, *cinR*, *expR*, *raiI*, and *raiR* mutants were quantified, confirming that the *cinS*, *cinI*, and *cinR* mutations enhanced the formation of biofilm rings (Fig. 6C). These enhanced biofilm rings were suppressed by cloned *cinS* (Fig. 6B, C), demonstrating that the enhanced biofilm rings in the *cinI* and *cinR* mutants are due to a lack of *cinS* expression, rather than a lack of CinI-made AHLs. The enhanced biofilm ring phenotype is independent of the *raiR* quorum-sensing system, because *raiI*



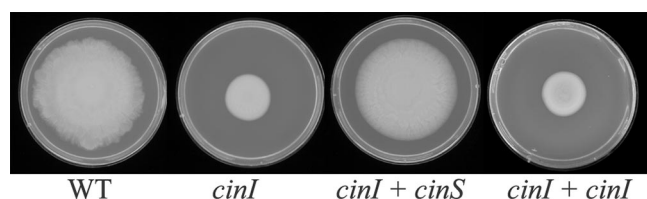


FIG. 7. Swarming by an *R. etli* *cinI* mutant was complemented by *cinS*, but not *cinI*. *R. etli* CNPAF512 (WT), FAJ4006 (*cinI*), and FAJ4006 containing cloned *cinS* on pIJ9692 (*cinI* + *cinS*) or cloned *cinI* on pIJ9655 (*cinI* + *cinI*) were spot inoculated onto the surfaces of YEM swarm plates, revealing that *cinS*, but not *cinI*, restored swarming to FAJ4006.

or *raiR* mutants did not produce an enhanced biofilm ring (Fig. 6C). The *expR* mutant A702 also produced an enhanced biofilm ring (Fig. 6), but it was less stable than that seen in the other mutants, resulting in high variation in the levels quantified (Fig. 6C); the enhanced biofilm in strain A702 (*expR*) was not suppressed by cloned *cinS* (Fig. 6C).

***cinS* regulates swarming in *R. etli*.** The predicted CinS sequence was used to search for related proteins using a TBLASTN search of the translated GenBank and EMBL DNA sequence databases. Closely related (several unannotated) proteins (Fig. 2B) were found in *R. leguminosarum* bv. *viciae* 3841, *R. leguminosarum* bv. *trifolii* strains WSM1325 and WSM2304, *Rhizobium etli* strains CFN42, CNPAF512, and CIAT652, and *Mesorhizobium tianshanense* (Fig. 2B). In each case, the coding region was immediately downstream from and apparently translationally coupled to *cinI*-like genes. In *R. etli*, mutation of *cinR* or *cinI* was previously shown to abolish swarming; the full swarming phenotype was not restored by the addition of the AHL made by CinI in that strain, but the AHL did induce a ruffling effect on the edge of the colony (10). On the basis of our results, it seemed likely that the *cinI* mutation was polar on *cinS*, which had not been recognized in that work as a separate gene cotranscribed with *cinI*. As shown (Fig. 7), cloned *cinS* (from *R. leguminosarum* strain 8401) fully restored swarming to the *R. etli* *cinI* mutant, but cloned *cinI* did not. This shows that the swarming must be regulated by CinS and not, as previously concluded (10) by contact with CinI-made AHLs; in addition, it shows that the mutation in *cinI* is polar on *cinS*. The *R. etli* *cinI* mutant carrying cloned *cinS* (pIJ9692) produced a swarm with an even edge, whereas the WT strain produced a swarm with an uneven edge (Fig. 7). Since the major difference is the presence of CinI-made AHLs in the WT, but not the mutant complemented with *cinS*, it is probable that the CinI-made AHLs induce the uneven edge on the swarming colony. We tested *R. leguminosarum* swarming under similar conditions but were unable to show *cinI*-dependent swarming clearly, and we did not see any difference between the control strain 8401 and the *cinI*, *cinR*, or *cinS* mutant derivatives of this strain, so an equivalent test of cloned *cinS* on swarming was not possible in that strain.

## DISCUSSION

In *Rhizobium* spp., *cinI*-*cinR*-determined quorum-sensing regulation is at the top of a hierarchy of regulation that includes the induction of the *raiI*-*raiR* genes (9, 52, 53). It is

evident from the work here that the CinI-CinR-mediated effect also requires both the LuxR-type regulator ExpR and *cinS*, a small gene immediately downstream of, and in the same operon as, *cinI*. It is clearly predicted that *cinS* encodes a protein, based on (i) the predicted translational coupling to *cinI* (which is also observed with all the orthologues shown in Fig. 2B), and (ii) the highly conserved amino acid sequences downstream of the variable N-terminal region (Fig. 2B).

The effect of the hierarchical regulation is to induce the expression of *raiR* in a population density-dependent manner, and a consequence of this is the increased expression of *raiI* and of AHLs produced by RaiI. The observations that cloned *cinS* can restore the decreased *raiR* expression in an *expR* mutant but that cloned *expR* cannot restore *raiR* expression in the *cinS* mutant indicate that CinS acts downstream of ExpR. ExpR is not required for the expression of *cinS*, because mutation of *expR* did not affect the expression of the promoter of the *cinIS* operon.

The *expR* gene identified here is homologous to *expR* from *S. meliloti*. The two predicted proteins are about 58% identical, and both are LuxR-type regulators encoded by genes located between the *ndvA* and *pyc* genes. In *S. meliloti* strain 1021, which is the most widely used strain for genetic studies, *expR* is interrupted by an endogenous IS element, a mutation which prevents the formation of an exopolysaccharide called EPS-II (35). ExpR induces the expression of EPS biosynthetic genes in response to C<sub>16:1</sub>-HSL, produced by SinI (32, 34). Mutation of *expR* or *sinI*, both of which are required for the production of EPS-II, alter the colony morphology of strains, and *expR* is required for full *sinI* induction, which also requires the LuxR-type regulator SinR- and SinI-made AHLs (32, 34). Whereas *expR* in *S. meliloti* is required for full *sinI* expression, *expR* in *R. leguminosarum* is not required for full *cinI* expression. Furthermore, it appears likely that, although it appears to have an AHL-binding domain, ExpR in *R. leguminosarum* can act independently of AHLs because, in a *cinI* *raiI* double mutant lacking detectable AHLs, cloned *cinS* restored full expression of *plyB*, which is ExpR regulated.

*R. leguminosarum* produces no polysaccharide equivalent to ExpR-regulated EPS-II made by *S. meliloti*, and the colony morphology of the *R. leguminosarum* *expR* mutant was very similar to the WT. In addition, CinI and CinR from *R. leguminosarum* show only about 30% identity to SinI and SinR from *S. meliloti*, as might be expected from nonorthologous AHL synthases and LuxR-type regulators. *R. leguminosarum* produces an acidic EPS structurally very different from the succinoglycan or EPS-II produced by *S. meliloti* (3), so there is not a clearly homologous target that ExpR is likely to regulate. The *expR* and *cinS* genes are needed for normal induction of *plyB*, which encodes a secreted glycanase (15, 16) that cleaves the acidic EPS (56). Since mutations in *raiI* and *raiR* had no effect on *plyB* expression, the *raiR* and *plyB* genes must be regulated in parallel, rather than *plyB* being regulated via *raiR*. There is a difference between *raiR* and *plyB* regulation, because although cloned *cinS* can restore *raiR* expression in an *expR* mutant, it cannot restore *plyB* expression in the *expR* mutant. The lack of *cinS* restoration of *plyB* expression in the *expR* mutant is consistent with the observation that the colony collapse phenotype induced by cloned *cinS* is absent or delayed in the *plyB* or *expR* mutants, possibly implying a more direct

role for ExpR in *plyB* expression compared with *raiR* expression.

Clearly, *cinS* can influence *raiR* expression and presumably other genes regulated by RaiR and surface EPS via its effects on *plyB* induction. Mutation of *plyB* or *cinI* in *R. etli* abolished swarming (5, 10) and since we have shown (Fig. 7) that the *cinI* mutation in *R. etli* is polar on *cinS*, this would be consistent with our proposed model of CinS playing a role in levels of *plyB* expression. Our data are not consistent with the proposal (10) that CinI-made AHLs have a direct effect on the extent of swarming by acting as a surfactant to reduce surface tension. It does appear that CinI-made AHLs may directly affect the characteristics of the swarm rather than its extent, because the swarming pattern in the *cinI* mutant carrying cloned *cinS* looked qualitatively different from that of the WT. It remains to be seen how many genes are regulated by CinS; in *S. meliloti*, ExpR and the *sinIR* quorum-sensing system regulate genes involved in motility and production of surface polysaccharides (24, 34).

We have not identified how CinS exerts its function. It is clear that, once expressed, CinS enhances *plyB* and *raiR* expression in the absence of CinI or RaiI-made AHLs, because cloned *cinS* restored *plyB* and *raiR* expression in a *raiI cinI* double mutant. Therefore, although CinI-made AHLs are normally required for *cinS* induction, they are not required for its activity, even though *cinI* and *cinS* are predicted to be translationally coupled in all strains in which they have been identified. There are no clear homologues of *cinS* outside the rhizobia, so it is difficult to predict how it acts. The most likely options are that CinS acts as a DNA- or RNA-binding protein that can enhance gene expression or RNA levels. There are several RNA-binding proteins that can influence quorum-sensing regulation in other bacteria (18, 22, 46, 48), sometimes circumventing the quorum-sensing regulatory system (21). However, there is also evidence for small DNA-binding proteins playing a role in quorum-sensing regulation (30) and a small quorum-sensing-induced protein (DegQ) that stimulates phosphotransfer to a transcriptional regulator that affects motility and biofilm formation (26). At this stage, we cannot easily determine whether any of these possibilities is likely, because CinS shows no similarity to any of these known regulators.

#### ACKNOWLEDGMENTS

We thank our colleagues Jim Lithgow, Adam Wilkinson, Vittoria Danino, Alan Williams, Fang Xie, and Daniela Russo for helpful discussions, and we thank Paul Williams and Siri Chhabra for making AHL standards available.

This work was supported in part by the Biotechnology and Biological Sciences Research Council via a grant-in-aid, a studentship (to J.J.), a grant (208/BRE13665) under the BIRE initiative, an award from CERES, and a contract (OLK3-CT-2000-31795) and a Marie Curie EST training award (019727) from the European Union.

#### REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Bagdasarian, M., R. Lurz, B. Ruckert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis. 1981. Specific-purpose plasmid cloning vectors: 2 broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**:237–247.
- Becker, A., and A. Puhler. 1998. Production of exopolysaccharides, p. 97–118. In H. P. Spink, A. Kondorosi, and P. J. J. Hooykaas (ed.), *The Rhizobiaceae*. Kluwer Academic, Dordrecht, The Netherlands.
- Beringer, J. E. 1974. R factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **84**:188–198.
- Braeken, K., R. Daniels, K. Vos, M. Fauvart, D. Bachaspatimayum, J. Vanderleyden, and J. Michiels. 2008. Genetic determinants of swarming in *Rhizobium etli*. *Microb. Ecol.* **55**:54–64.
- Buchanan-Wollaston, A. V. 1979. Generalised transduction in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **112**:135–142.
- Cha, C., P. Gao, Y. C. Chen, P. D. Shaw, and S. K. Farrand. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interact.* **11**:1119–1129.
- Cubo, M. T., A. Economou, G. Murphy, A. W. Johnston, and J. A. Downie. 1992. Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABC* that can influence nodulation by *Rhizobium leguminosarum* biovar viciae. *J. Bacteriol.* **174**:4026–4035.
- Daniels, R., D. E. De Vos, J. Desair, G. Raedschelders, E. Luyten, V. Rosemeyer, C. Verreth, E. Schoeters, J. Vanderleyden, and J. Michiels. 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. *J. Biol. Chem.* **277**:462–468.
- Daniels, R., S. Reynaert, H. Hoekstra, C. Verreth, J. Janssens, K. Braeken, M. Fauvart, S. Beullens, C. Heusdens, I. Lambrichts, D. E. De Vos, J. Vanderleyden, J. Vermant, and J. Michiels. 2006. Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *Proc. Natl. Acad. Sci. USA* **103**:14965–14970.
- Daniels, R., J. Vanderleyden, and J. Michiels. 2004. Quorum sensing and swarming migration in bacteria. *FEMS Microbiol. Rev.* **28**:261–289.
- Danino, V. E., A. Wilkinson, A. Edwards, and J. A. Downie. 2003. Recipient-induced transfer of the symbiotic plasmid pRL11 in *Rhizobium leguminosarum* bv. viciae is regulated by a quorum-sensing relay. *Mol. Microbiol.* **50**:511–525.
- Downie, J. A., and J. E. Gonzalez. 2008. Cell-to-cell communication in rhizobia: quorum sensing and plant signalling, p. 213–232. In S. C. Winans and B. L. Bassler (ed.), *Chemical communication among bacteria*. ASM Press, Washington, DC.
- Downie, J. A., C. D. Knight, A. W. B. Johnston, and L. Rossen. 1985. Identification of genes and gene-products involved in the nodulation of peas by *Rhizobium leguminosarum*. *Mol. Gen. Genet.* **198**:255–262.
- Finnie, C., N. M. Hartley, K. C. Findlay, and J. A. Downie. 1997. The *Rhizobium leguminosarum* *prsDE* genes are required for secretion of several proteins, some of which influence nodulation, symbiotic nitrogen fixation and exopolysaccharide modification. *Mol. Microbiol.* **25**:135–146.
- Finnie, C., A. Zorreguieta, N. M. Hartley, and J. A. Downie. 1998. Characterization of *Rhizobium leguminosarum* exopolysaccharide glycanases that are secreted via a type I exporter and have a novel heptapeptide repeat motif. *J. Bacteriol.* **180**:1691–1699.
- Glenn, S. A., N. Gurich, M. A. Feeney, and J. E. Gonzalez. 2007. The ExpR/Sin quorum-sensing system controls succinoglycan production in *Sinorhizobium meliloti*. *J. Bacteriol.* **189**:7077–7088.
- Gonzalez, N., S. Heeb, C. Valverde, E. Kay, C. Reimann, T. Junier, and D. Haas. 2008. Genome-wide search reveals a novel GacA-regulated small RNA in *Pseudomonas* species. *BMC Genomics* **9**:167.
- Gonzalez, V., R. I. Santamaria, P. Bustos, I. Hernandez-Gonzalez, A. Medrano-Soto, G. Moreno-Hagelsieb, S. C. Janga, M. A. Ramirez, V. Jimenez-Jacinto, J. Collado-Vides, and G. Davila. 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. *Proc. Natl. Acad. Sci. USA* **103**:3834–3839.
- Gray, K. M., J. P. Pearson, J. A. Downie, B. E. Boboye, and E. P. Greenberg. 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. *J. Bacteriol.* **178**:372–376.
- Hammer, B. K., and B. L. Bassler. 2007. Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **104**:11145–11149.
- Heeb, S., S. A. Kuehne, M. Bycroft, S. Crivii, M. D. Allen, D. Haas, M. Camara, and P. Williams. 2006. Functional analysis of the post-transcriptional regulator RsmA reveals a novel RNA-binding site. *J. Mol. Biol.* **355**:1026–1036.
- Hoang, H. H., A. Becker, and J. E. Gonzalez. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J. Bacteriol.* **186**:5460–5472.
- Hoang, H. H., N. Gurich, and J. E. Gonzalez. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. *J. Bacteriol.* **190**:861–871.
- Knight, C. D., L. Rossen, J. G. Robertson, B. Wells, and J. A. Downie. 1986. Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nodABC* genes and analysis of early stages of plant infection. *J. Bacteriol.* **166**:552–558.
- Kobayashi, K. 2007. Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol. Microbiol.* **66**:395–409.
- Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. 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.
28. Lamb, J. W., J. A. Downie, and A. W. Johnston. 1985. Cloning of the nodulation (*nod*) genes of *Rhizobium phaseoli* and their homology to *R. leguminosarum nod* DNA. *Gene* **34**:235–241.
  29. Lamb, J. W., G. Hombrecher, and A. W. B. Johnston. 1982. Plasmid-determined nodulation and nitrogen-fixation abilities in *Rhizobium phaseoli*. *Mol. Gen. Genet.* **186**:449–452.
  30. Lenz, D. H., and B. L. Bassler. 2007. The small nucleoid protein Fis is involved in *Vibrio cholerae* quorum sensing. *Mol. Microbiol.* **63**:859–871.
  31. 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.
  32. Marketon, M. M., S. A. Glenn, A. Eberhard, and J. E. González. 2003. Quorum sensing controls exopolysaccharide production in *Sinorhizobium meliloti*. *J. Bacteriol.* **185**:325–331.
  33. 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. Stewart, and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acyl-homoserine lactones. *Microbiology* **143**:3703–3711.
  34. McIntosh, M., E. Krol, and A. Becker. 2008. Competitive and cooperative effects in quorum-sensing-regulated galactoglucan biosynthesis in *Sinorhizobium meliloti*. *J. Bacteriol.* **190**:5308–5317.
  35. Pellock, B. J., M. Teplitski, R. P. Boinay, W. D. Bauer, and G. C. Walker. 2002. A LuxR homolog controls production of symbiotically active extracellular polysaccharide II by *Sinorhizobium meliloti*. *J. Bacteriol.* **184**:5067–5076.
  36. Prentki, P., and H. M. Krisch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303–313.
  37. Quandt, J., and M. F. Hynes. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**:15–21.
  38. Rodelas, B., J. K. Lithgow, F. Wisniewski-Dye, A. Hardman, A. Wilkinson, A. Economou, P. Williams, and J. A. Downie. 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*. *J. Bacteriol.* **181**:3816–3823.
  39. 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.
  40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
  41. Sharma, S. B., and E. R. Signer. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-gusA. *Genes Dev.* **4**:344–356.
  42. Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **94**:6036–6041.
  43. Sherwood, M. T. 1970. Improved synthetic medium for the growth of *Rhizobium*. *J. Appl. Bacteriol.* **33**:708–713.
  44. Sourjik, V., P. Muschler, B. Scharf, and R. Schmitt. 2000. VisN and VisR are global regulators of chemotaxis, flagellar, and motility genes in *Sinorhizobium (Rhizobium) meliloti*. *J. Bacteriol.* **182**:782–788.
  45. Spaink, H. P., C. A. Wijffelman, E. Pees, R. J. H. Okker, and B. J. J. Lugtenberg. 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. *Nature* **328**:337–340.
  46. Svenningsen, S. L., C. M. Waters, and B. L. Bassler. 2008. A negative feedback loop involving small RNAs accelerates *Vibrio cholerae* transition out of quorum-sensing mode. *Genes Dev.* **22**:226–238.
  47. Thorne, S. H., and H. D. Williams. 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. *phaseoli*: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. *J. Bacteriol.* **181**:981–990.
  48. Tu, K. C., and B. L. Bassler. 2007. Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev.* **21**:221–233.
  49. Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond. 2001. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25**:365–404.
  50. Wilkinson, A., V. Danino, F. Wisniewski-Dye, 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.
  51. Winans, S. C. 2008. Cell-cell signalling within crown gall tumors, p. 291–306. In S. C. Winans and B. L. Bassler (ed.), *Chemical communication among bacteria*. ASM Press, Washington, DC.
  52. Wisniewski-Dye, F., and J. A. Downie. 2002. Quorum-sensing in *Rhizobium*. *Antonie van Leeuwenhoek* **81**:397–407.
  53. Wisniewski-Dye, F., J. Jones, S. R. Chhabra, and J. A. Downie. 2002. *raiIR* genes are part of a quorum-sensing network controlled by *cinI* and *cinR* in *Rhizobium leguminosarum*. *J. Bacteriol.* **184**:1597–1606.
  54. Young, J. P. W., L. C. Crossman, A. W. B. Johnston, N. R. Thomson, Z. F. Ghazoui, K. H. Hull, M. Wexler, A. R. J. Curson, J. D. Todd, P. S. Poole, T. H. Mauchline, A. K. East, M. A. Quail, C. Churcher, C. Arrowsmith, I. Cherevach, T. Chillingworth, K. Clarke, A. Cronin, P. Davis, A. Fraser, Z. Hance, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabinowitz, M. Sanders, M. Simmonds, S. Whitehead, and J. Parkhill. 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biology* **7**:R34.
  55. Zhu, J., P. M. Oger, B. Schrammeijer, P. J. Hooykaas, S. K. Farrand, and S. C. Winans. 2000. The bases of crown gall tumorigenesis. *J. Bacteriol.* **182**:3885–3895.
  56. Zorreguieta, A., C. Finnie, and J. A. Downie. 2000. Extracellular glycanases of *Rhizobium leguminosarum* are activated on the cell surface by an exopolysaccharide-related component. *J. Bacteriol.* **182**:1304–1312.