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The *cin* and *rai* Quorum-Sensing Regulatory Systems in *Rhizobium leguminosarum* Are Coordinated by ExpR and CinS, a Small Regulatory Protein Coexpressed with CinI▽

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To understand how the *Rhizobium leguminosarum raiI-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-$C_{14:1}$-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_8-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).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source
R. leguminosarum strains		
8400	Strain lacking a symbiotic plasmid	29
8401	Str^r derivative of strain 8400	29
A34	Strain 8401 containing pRL1JI (pSym)	14
A616	8401 *prsD1*::Tn*5*	16
A552	8401 *cinR1*::Tn*5*	31
A702	8401 *expR1*::Tn*5*-gus	This work
A740	8401 *cinI3*::Spc^r	31
A789	8401 *raiI7*::Tn*5*	53
A797	8401 *cinI3*::Spc^r *raiI7*::Tn*5*	53
A802	8401 *raiR8*::Tn*5*	53
A1085	8400 *expR1*::Tn*5*-gus	This work
A1102	8401 carrying *cinS*::Spc^r	This work
R. etli CNPAF512	WT *R. etli*	10
R. etli FAJ4006	CNPAF512 *cinI*::Tn*5*-gus	10
A. tumefaciens NT1/pZLR4	*traG-lacZ*-based AHL detection strain	7
C. violaceum CV026	AHL detection strain	33
Plasmids		
pIJ7516	Cosmid carrying *cinRI* locus	31
pIJ7910	*cinI′-lacZ* in pMP220	31
pIJ9001	Cosmid carrying *raiIR* region	53
pIJ9115	7-kb EcoRI fragment from strain A702 carrying part of the Tn*5*-gus in pBluescript SK	This work
pIJ9123	Cosmid carrying *expR* region in pLAFR1	This work
pIJ9229	*expR* on 3-kb EcoRI fragment from pIJ9123 in pBluescript KS	This work
pIJ9252	*plyB′-lacZ* fusion in pMP220	This work
pIJ9263	*expR′-lacZ* fusion in pMP220	This work
pIJ9272	*raiR′-lacZ* fusion in pMP220	53
pIJ9276	*raiR* in pBBR1-MCS5	53
pIJ9280	*raiI′-lacZ* fusion in pMP220	53
pIJ9493	*expR* in pBBR1-MCS5	This work
pIJ9655	*cinI* and truncated *cinS* in pKT230	This work
pIJ9692	*cinS* in pKT230	This work
pIJ9769	*expR* in pBBR1-MCS2	This work
pMP220	Broad-host-range *lacZ* expression vector; Tc^r	45
pKT230	Broad-host-range *lacZ* expression vector; Kan^r	2
pBBR1-MCS2	Broad-host-range plasmid; Kan^r	27
pBBR1-MCS5	Broad-host-range plasmid; Gent^r	27

The *cinI* and *cinR* quorum-sensing genes control induction of the *traI* and *traR* quorum-sensing regulons via CinI-made 3-OH-$C_{14:1}$-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 *raiI* and *raiR* expression has not been established. In this work we demonstrate that *raiI* and *raiR* 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 β-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_{600}) 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_2O, 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_{575} 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 Tn*5*-gus by using *Escherichia coli* strain MM294/pRK600::Tn*5*-gus as a donor of the suicide plasmid pRK600::Tn*5*-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-$C_{14:1}$-HSL. Strain A1085 was made by plating bacteriophage RL38 (6) on strain A702 and using the phage lysate to transduce strain 8400, selecting for kanamycin resistance. To make strain A1102 (*cinS*::Spc^r), *cinS* with 1-kb flanking regions was first amplified by PCR using primers CTGAAGA**GCGGCCGC**TTCAAGCTC and GAGAAACTTA**GCGGC CGC**TATTGATTTC containing introduced NotI sites (bold face). The product

was digested with NotI and cloned into pJQ254 (37). The *cinS1*::Spc^r allele was then created by cloning a spectinomycin resistance cassette on a BamHI fragment from pHP45Ω (36) into the unique BamHI site in *cinS*. The 4-kb NotI fragment containing *cinS1*::Spc^r was then subcloned into pJQ200 (37), and the *cinS1*::Spc^r allele was recombined into strain 8401 by selecting for spectinomycin-resistant, sucrose-resistant transconjugants (37).

Molecular biology techniques and plasmid construction. DNA work was done using standard methods (40). Plasmid pIJ9115 was made by cloning the kanamycin resistance cassette from the genome of A702 as a 7-kb EcoRI fragment in pBluescript SK. Plasmid pIJ9123 was isolated from a cosmid library (28) on the basis of its ability to complement strain A702 for AHL production using *C. violaceum* CV026 as assayed previously (53). A 3-kb EcoRI fragment containing *expR* was subcloned from pIJ9123 into pBluescript to form pIJ9229, and pIJ9263 (*expR′-lacZ*) was made by cloning a 1.4-kb EcoRI-PstI fragment from pIJ9123 into pMP220. To construct plasmid pIJ9252 (*plyB′-lacZ*), a 0.5-kb EcoRI-PstI fragment from pIJ7708 (16) was cloned into the EcoRI-PstI sites of pMP220. To make plasmid pIJ9493, *expR* was subcloned from pIJ9229 on a 2.7-kb EcoRI-SacI fragment into pBBR1-MCS5, and *expR* was recloned on a 2.6-kb HindIII-SacI fragment from pIJ9493 into pBBR1-MCS2 to make pIJ9769. To make pIJ9655, *cinI* and a truncated *cinS* (encoding the first 36 residues) was amplified from pIJ7655 (31) by PCR using primers CATTCTG**GGATCC**ACGAACTTGA AAAC and ATCT**GAATCC**CGATCTGCGTG which carry introduced BamHI and EcoRI sites (shown in boldface type); the product was cloned into pKT230 using BamHI and EcoRI. To make pIJ9692, *cinS* was amplified from plasmid pIJ7655 (31) using the forward primer CTTC**GAATT**CTCGGACCGCGTGCT GCGCAAGATG and the reverse primer GATATCGACCAT**GAATTC**CCTG ATGAC containing introduced EcoRI sites (boldface); the product was digested with EcoRI and cloned as a 305-bp fragment into the EcoRI site in pKT230 in the same orientation as the streptomycin resistance gene in that plasmid. All plasmid inserts made using PCR were checked by DNA sequencing.

DNA sequencing of the region containing *expR* (accession no. FM992852) was carried out on both strands using primer walking on pIJ9229. The location of Tn*5*-gus in *expR* was determined by sequencing pIJ9115 using a Tn*5*-specific primer. Database searches of the predicted protein sequences were carried out by using the BLAST and FASTA (1) programs to find related sequences in the EMBL and SwissProt protein sequence databases.

AHL assays. *R. leguminosarum* cultures were grown for 48 h in TY medium to an OD_{600} of about 1.0, and cells were removed by centrifugation. AHLs were extracted from culture supernatants and analyzed by thin-layer chromatography (31) using *Agrobacterium tumefaciens* NT1/pZLR4 (7, 42). The amount loaded corresponds to the extract from 1 ml of culture supernatant. CinI-made 3-OH-$C_{14:1}$-HSL was bioassayed on TY medium (50).

RESULTS

Identification of a regulatory gene (*expR*) required for *raiI* expression. To identify genes required for *raiI* and *raiR* expression, *R. leguminosarum* strain 8401 (lacking a symbiotic plasmid) mutants were screened for decreased levels of RaiI-made AHLs. We used *C. violaceum* strain CV026, which produces a purple pigment (violacein) in response to RaiI (but not CinI)-made AHLs (33, 53). Strain A702 produced low levels of AHLs, and the mutation was not in the *raiIR* or *cinIR* region on the basis of the lack of complementation with pIJ9001 and pIJ7516, respectively. Analysis of A702-made AHLs by thin-layer chromatography using *A. tumefaciens* carrying *traG-lacZ* to detect AHLs revealed that, in contrast with the wild type (WT) (strain 8401) (Fig. 1A, lane 2), strain A702 produced no detectable C_8-HSL and a very low level of 3-OH-C_8-HSL (Fig. 1A, lane 3), slightly more than that made by a *raiR* mutant (Fig. 1A, lane 4), which lacks *raiI* expression (53). The A702 mutant phenotype cotransduced (100%) with kanamycin resistance, showing that the transposon mutation in A702 caused the loss of AHL production. The transductant A1085 behaved as strain A702 did.

A cosmid (pIJ9123) that restored AHL production to strain A702 was identified and used to probe EcoRI-digested genomic DNA from A702 and the control strain 8401, revealing that the transposon in A702 had inserted in a 3-kb EcoRI fragment. This fragment was cloned from pIJ9123 and partially sequenced, revealing the *expR* gene between *ndvA* and *pyc*. *expR* is transcribed divergently from *ndvA* and in the same orientation as *pyc*. The transposon in strain A702 was inserted 678 bp downstream of the predicted translation start of *expR*. The *ndvA expR pyc* gene arrangement is similar to that seen in *R. leguminosarum* 3841 (54), *R. etli* CFN42 (19), and *S. meliloti* strain 1021 (http://sequence.toulouse.inra.fr/meliloti.html), except that in strain 1021, there is an insertion element within *expR* (35). *expR* encodes a predicted LuxR-type regulator with predicted N-terminal AHL-binding and C-terminal DNA-binding domains. Cloned *expR* (on pIJ9493) complemented AHL production in strain A702 (*expR*) (Fig. 1A, lane 5) but did not suppress the defect in AHL production in the *raiR* mutant

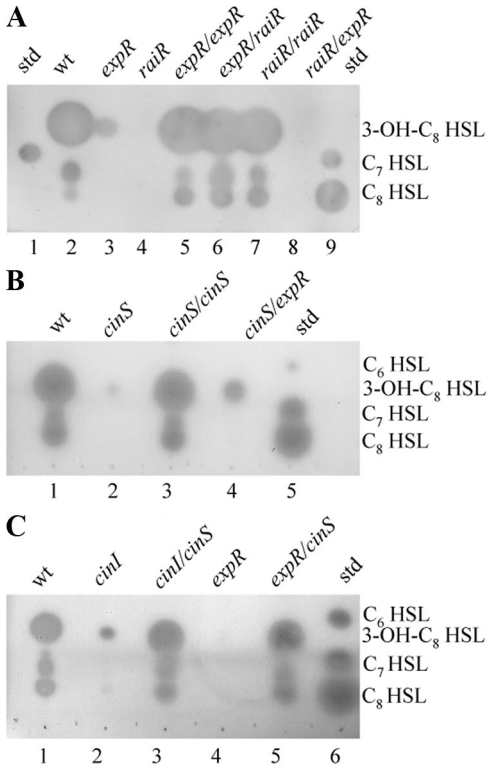
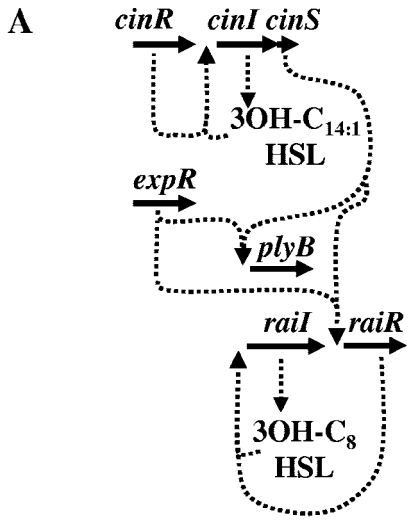


FIG. 1. Effects of cloned *expR*, *raiR*, and *cinS* on production of AHLs by *R. leguminosarum* quorum-sensing mutants. AHLs were extracted from cell-free culture supernatants, separated by thin-layer chromatography, and detected using an overlay of agar seeded with the biosensor *A. tumefaciens* NT1/pZLR4. Each lane was loaded with a volume of extract equivalent to 1 ml of culture. (A) Lane 1, C_7-HSL standard (std) (14 pmol); lane 2, strain 8401 (WT); lane 3, strain A702 (*expR*); lane 4, strain A802 (*raiR*); lane 5, strain A702 (*expR*) carrying pIJ9493 (*expR*); lane 6, A702 (*expR*) carrying pIJ9276 (*raiR*); lane 7, A802 (*raiR*) carrying pIJ9276 (*raiR*); lane 8, A802 (*raiR*) carrying pIJ9493 (*expR*); lane 9, standards, i.e., C_8-HSL (100 pmol) and C_7-HSL (14 pmol). (B) Lane 1, strain 8401 (WT); lane 2, strain A1102 (*cinS*); lane 3, A1102 (*cinS*) carrying pIJ9692 (*cinS*); lane 4, A1102 (*cinS*) carrying pIJ9493 (*expR*); lane 5, standards, i.e., C_8-HSL (100 pmol), C_7-HSL (14 pmol), and C_6-HSL (5 pmol). (C) Lane 1, strain 8401 (WT), lane 2, strain A740 (*cinI*); lane 3, strain A740 (*cinI*) carrying pIJ9692 (*cinS*); lane 4, A1085 (*expR*); lane 5, A1085 (*expR*) carrying pIJ9692 (*cinS*); lane 6, standards, i.e., C_8-HSL (100 pmol), C_7-HSL (14 pmol), and C_6-HSL (5 pmol).



B
CinS:

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MNRLAETATQTSMQKDIGALERHVLSARDIATQI
(a)          AI
(b)        ----I
(c)      T  ----    S
(d) V      P----    S
(e)      T   AI

GDPFLSYLLSMALFTIYEKKAHHENEALKSSFS
(c,d)                       Q
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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 (±300) units of β-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-$C_{14:1}$-HSL, we added 1 μM 3-OH-$C_{14:1}$-HSL to the growth medium of the *cinI* mutant A740 carrying *raiR′-lacZ* (pIJ9272) but saw no induction (236 ± 37 units); we could not restore *raiI* or *raiR* expression in the *cinI* mutant with other AHLs, including C_6-HSL, 3-O-C_8-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-$C_{14:1}$-HSL (data not shown), and A1102/pIJ7910 (*cinI′-lacZ*) had normal levels of β-galactosidase expression (20,628 ± 2,694 units compared with 24,692 ± 1,940 units seen with the control 8401/pIJ7910). This contrasts with the relatively low expression (986 ± 75 units) in the *cinI* mutant A740 carrying pIJ7910. 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 ± 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[a]

Strain (relevant genotype)	Expression[b] of:	
	raiI′-lacZ (pIJ9280)	*raiR′-lacZ* (pIJ9272)
8401 (WT)	5,873 ± 10	1,075 ± 146
A702 (*expR*)	340 ± 25	282 ± 44
A740 (*cinI*)	346 ± 18	302 ± 53
A1102 (*cinS*)	362 ± 9	318 ± 30
A789 (*raiI*)	462 ± 16	1,135 ± 99
A802 (*raiR*)	344 ± 13	1,030 ± 7

[a] Bacteria were cultured for 48 h in Y-mannitol medium.
[b] Data shown are mean values for β-galactosidase activity (in Miller units) ± standard errors, based on averages of at least six assays.

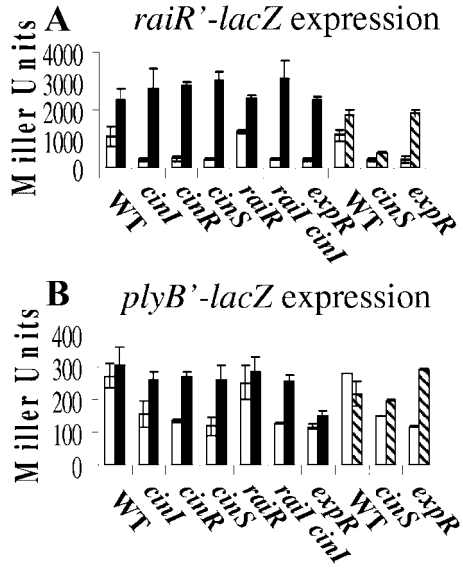

FIG. 3. Effects of cloned *cinS* and *expR* on *raiR'-lacZ* and *plyB'-lacZ* expression in quorum-sensing mutants of *R. leguminosarum*. Strains were grown in Y-mannitol medium, and β-galactosidase activity was assayed after 48 h (for *raiR'-lacZ*) or 72 h (for *plyB'-lacZ*) of growth. The WT strain (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 pKT230 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, pKT230 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 ± 9 units compared with 4,089 ± 347 units in WT strain 8401) and was not affected by cloned *cinS* (79 ± 18 units), showing that RaiR 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 ± 1,753 units compared with 24,692 ± 1,940 units in strain 8401/pIJ7910) and produced normal levels of CinI-made 3-OH-$C_{14:1}$-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 ± 45 units compared with 902 ± 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 ± 56 units), A789 (*raiI*) (813 ± 119 units), A797 (*raiI cinI*) (865 ± 86 units), and A802 (*raiR*) (732 ± 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

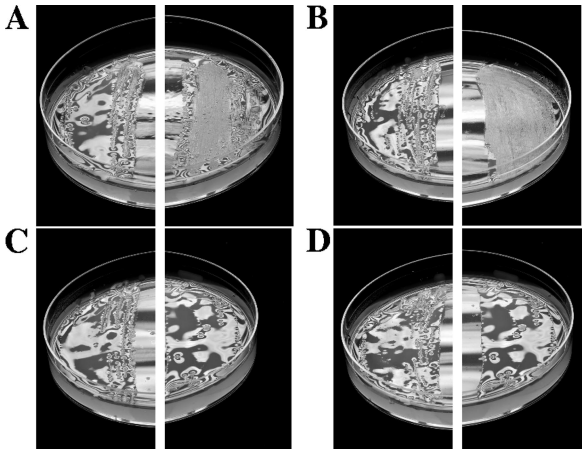

FIG. 4. Cloned *cinS* induced a colony collapse phenotype. The photographs show cultures of strains grown for 3 days on TY agar medium. For each strain, the photograph on the left shows the strain grown with the vector (pKT230) control and the photograph on the right shows the strain with *cinS* on pIJ9692. 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

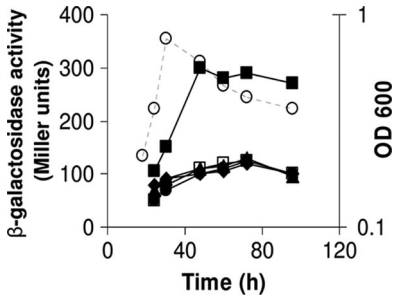

FIG. 5. Decrease of *plyB-lacZ* expression in *cinI*, *cinR*, *cinS*, and *expR* mutants. Expression of *plyB* was assayed throughout growth in Y-mannitol medium using pIJ9252 (*plyB′-lacZ*) in the WT strain 8401 (solid squares), the *cinI* mutant A740 (solid triangles), the *cinR* mutant A552 (solid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

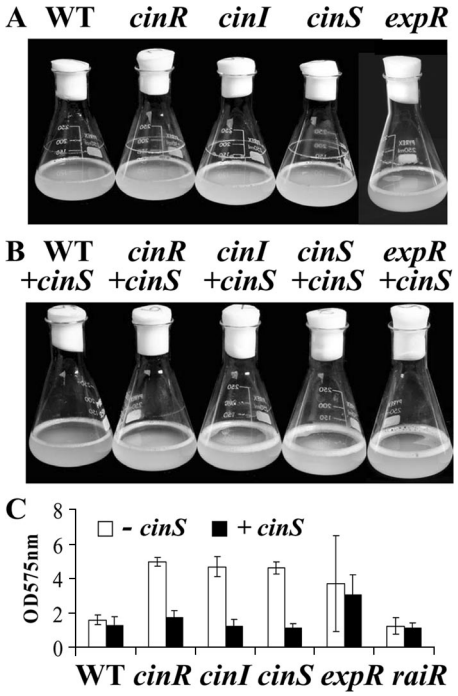

FIG. 6. Effects of cloned *cinS* on biofilm rings produced by quorum-sensing mutants. (A) Biofilm rings formed at the air-liquid interface of shaking flask cultures of strains grown for 5 days in Y-mannitol medium are shown. Strains A552 (*cinR*), A740 (*cinI*), A1102 (*cinS*), and A702 (*expR*) produced 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 *raiIR* quorum-sensing system, because *raiI*

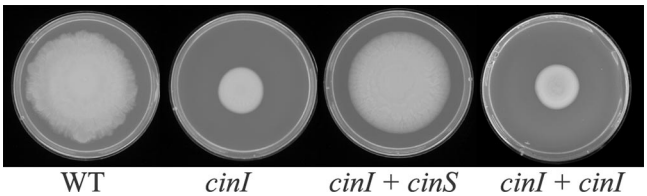

FIG. 7. Swarming by an *R. etli cinI* mutant was complemented by *cinS*, but not *cinI*. *R. etli* CNPAF512 (WT), FAJ4006 (*cinI*), and FAJ4006 containing cloned *cinS* on pIJ9692 (*cinI* + *cinS*) or cloned *cinI* on pIJ9655 (*cinI* + *cinI*) were spot inoculated onto the surfaces of YEM swarm plates, 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 $C_{16:1}$-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.

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