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The Quorum Sensing Regulator CinR Hierarchically Regulates Two Other Quorum Sensing Pathways in Ligand-Dependent and -Independent Fashions in *Rhizobium etli*

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ABSTRACT

Many rhizobial species use complex *N*-acyl-homoserine lactone (AHL)-based quorum sensing (QS) systems to monitor their population density and regulate their symbiotic interactions with their plant hosts. There are at least three LuxRI-type regulatory systems in *Rhizobium etli* CFN42: CinRI, RaiRI, and TraRI. In this study, we show that CinI, RaiI, and TraI are responsible for synthesizing all AHLs under the tested conditions. The activation of these AHL synthase genes requires their corresponding LuxR-type counterparts. We further demonstrate that CinRI is at the top of the regulatory cascade that activates RaiRI and TraRI QS systems. Moreover, we discovered that CinR possesses a specific affinity to bind *cinI* promoter in the absence of its cognate AHL ligand, thereby activating *cinI* transcription. Addition of AHLs leads to improved binding to the *cinI* promoter and enhanced *cinI* expression. Furthermore, we found that compared to the wild type, the *cinR* mutation displayed reduced nodule formation, and *cinR*, *raiR*, and *traI* mutants show significantly lower levels of nitrogen fixation activity than the wild type. These results suggest that the complex QS regulatory systems in *R. etli* play an important role in its symbiosis with legume hosts.

IMPORTANCE

Many bacteria use quorum sensing (QS) to monitor their cell densities and coordinately regulate a number of physiological functions. Rhizobia often have diverse and complex LuxR/LuxI-type quorum sensing systems that may be involved in symbiosis and N_2 fixation. In this study, we identified three LuxR/LuxI-type QS systems in *Rhizobium etli* CFN42: CinRI, RaiRI, and TraRI. We established a complex network of regulation between these QS components and found that these QS systems played important roles in symbiosis processes.

Establishment of symbiosis between rhizobia and their legume hosts is a complex process requiring multiple intricate signal exchanges. Many plant-associated bacteria, such as species of legume-nodulating rhizobia, use a set of diffusible *N*-acyl homoserine lactones (AHLs) involved in quorum sensing (QS) (1) systems to communicate with each other and optimize their interactions with plant hosts. In a typical complete LuxRI QS system, AHLs are synthesized by an LuxI-type protein and are accumulated to threshold levels as cell density increases. An LuxR-type transcriptional activator is activated by threshold levels of AHLs and induces expression of specific target genes to regulate multiple physiological functions. Quorum sensing has been implicated in various aspects of legume symbioses, including exopolysaccharide production, which is important for infection, plasmid transfer, competitiveness, nodule formation, and nitrogen fixation (2).

Quorum sensing in rhizobia is very diverse, and two strains from the same species of rhizobia often do not have the same quorum sensing components (3). For example, in *Sinorhizobium meliloti*, SinR/SinI and ExpR in strain Rm1021 regulate exopolysaccharide production and swarming (4–6), TraR/TraI in Rm41 controls plasmid transfer (2, 6), and VisN/VisR in RU10/406 regulates motility (7). In the *Rhizobium etli* strain CNPAF512, two QS systems have been identified, RaiRI and CinRI, both of which control symbiosis (8, 9). The *raiI* mutants cause a slight increase in the number of nodules formed per plant (9), whereas mutations in *cinI* exhibit a 60 to 70% reduction in nitrogen fixation efficiency (8). Another *R. etli* strain, CFN42, has a complex but less well characterized QS system (10, 11). There are three different LuxI-type AHL synthases (CinI, RaiI, and TraI) and four cognate LuxR-type regulators (CinR, RaiR, TraR1, and TraR2) in the genome sequence (Fig. 1A). Only one *N*-acyl-homoserine lactone, *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL), synthesized by TraI, has been identified. Together with its two activators, TraR and the adjacent CinR, 3-oxo-C_8-HSL has been found to be involved in self-conjugative plasmid transfer (12). In order to fully understand the quorum sensing regulation in *R. etli* CFN42, we constructed mutations in each of the aforementioned QS regulatory components and studied the effects of these mutants on transcription of QS regulatory genes as well as AHL production. Interest-

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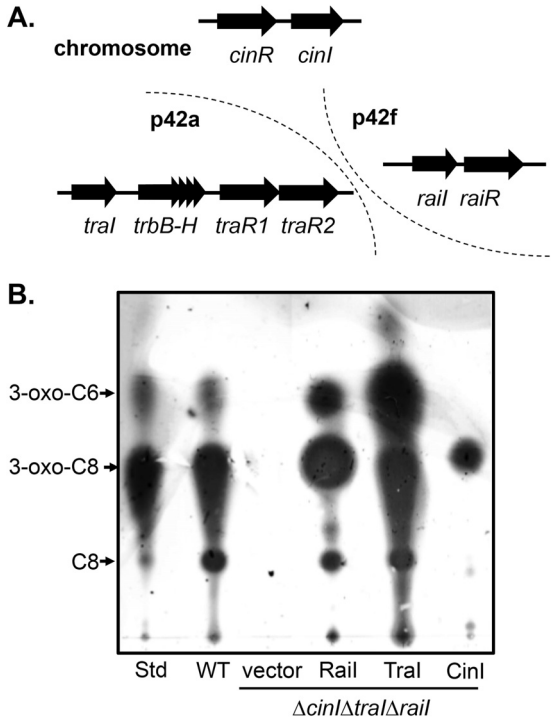

FIG 1 AHL production in *R. etli* CFN42. (A) Genomic location and organization of *R. etli* QS systems. p42f and p42a are two plasmids in *R. etli* CFN42. (B) TLC analysis. Cell-free supernatants of *R. etli* wild-type and Δ*cinI* Δ*raiI* Δ*traI* mutant strains harboring either the vector control, P_{tac}-*raiI* (RaiI), P_{tac}-*traI* (TraI), or P_{tac}-*cinI* (CinI) plasmid were subjected to TLC analysis. Each equivalent of 1 ml of culture extract was loaded on a C_{18} reverse-phase TLC plate, followed by an overlay of agar medium seeded with AHL bioassay strains (16). An extract from *A. tumefaciens* R10(pCF218) was used as the standard, and the migration positions of 3-oxo-C_8, 3-oxo-C_6, and C_8-HSL, as indicated, were based on a previously published TLC analysis (16, 38). WT, wild type.

ingly, we found that CinR can activate *cinI* expression in the absence of its ligand, thereby demonstrating the complexity of the QS regulatory pathways. The relationship between QS and symbiosis is also investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Rhizobium etli* CFN42 streptomycin-resistant CE3 derivative strains were cultured at 28°C in peptone-yeast extract (PY) medium (13), *Escherichia coli* was cultured at 37°C in Luria-Bertani (LB) medium, and *Agrobacterium tumefaciens* was grown at 28°C in AT medium (1). The following antibiotics were added as appropriate to maintain selection for plasmids (final concentration): streptomycin (100 μg/ml), gentamicin (20 μg/ml), spectinomycin (100 μg/ml), and tetracycline (2 μg/ml). When required, medium was supplemented with 1.5% agar. To construct the in-frame deletion mutants, overlap extension PCR was first used to create the deletions. In-frame deletion plasmids in the *cinI*, *cinR*, *raiI*, *raiR*, *traI*, *traR*, *traR2*, and *traR1-traR2* genes were constructed by overlapping PCR of flanking regions of the target genes and cloning the resulting product in the pEX18Gm suicide vector containing an *sacB* counterselectable marker (14). The resulting plasmids were introduced into *R. etli* by conjugation, and deletion mutants were selected for double homologous recombination events. Double-crossover events were selected on sucrose plates (10%) after the first "cross-in" homologous recombination. The deletions in each mutant strain were confirmed by PCR examination and sequence analysis. Each plasmid that constitutively expressed *cinI*, *cinR*, *raiI*, *raiR*, *traI*, and *traR1-traR2* was constructed by cloning these genes into the pYC12 vector (15), and constructs were introduced into *R. etli* and *E. coli* strains by electroporation. Primers used in this study are listed in Table S1 in the supplemental material.

AHL bioassays and identification. Spent medium (2%, vol/vol) from *R. etli* strains grown in PY medium were collected at various time points as indicated in Fig. 2 and added into AT medium (1) (optical density at 600 nm of 0.2 to 0.8), and approximately 10^7 cells per ml of AHL bioassay strain were added. We used *A. tumefaciens* KYC55(pJZ372)(pJZ384)(pJZ410) as the AHL bioassay strain (16). This strain cannot make its own AHLs, and the AHL receptor TraR of *A. tumefaciens* is overexpressed such that it sensitively detects exogenous AHLs with diverse structures and activates the *traI-lacZ* reporter. These cultures were incubated with aeration for 12 h and assayed for β-galactosidase-specific activity (17).

Thin-layer chromatography (TLC) analysis of AHLs released by *R. etli* strains was performed as previously described (18). *R. etli* was grown for 24 h in 50 ml of medium. After centrifugation, the supernatant was extracted twice with an equal volume of dichloromethane. The extract was evaporated to dryness by vacuum rotation at 35°C and redissolved in 50 μl of dichloromethane. One microliter of this suspension was spotted on a C_{18} reverse-phase thin-layer chromatography (TLC) plate (RP-18F_{254S}; Merck), which was then developed with 60% methanol. The plate was removed from the chromatography tank and dried in air for 4 h. The air-dried chromatography plate was overlaid with a thin film of AT medium soft agar (1% [wt/vol]) containing 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) and seeded with *A. tumefaciens* KYC55(pJZ372)(pJZ384)(pJZ410) indicator cells (16). The plate was incubated at 28°C for 12 to 16 h and examined for X-Gal hydrolysis.

Transcriptional analysis. LacZ fusion reporters of *cinR*, *raiR*, *traR*, *cinI*, *raiI*, and *traI* were constructed by cloning promoter sequences of the genes of interest (~0.5-kb sequences upstream of the start codon and ~30 bp into the reading frame) into pRA302 (19), which contains a promoterless *lacZ* reporter. The coding sequence of the gene of study was fused in frame with the *lacZ* reading frame. The resulting plasmids were introduced into *R. etli* wild type and various QS mutants by electroporation. Bacteria containing *lacZ* translational reporters were grown in PY medium with appropriate antibiotics in the absence and in the presence of the AHL indicated. We tested the samples at the time points indicated in the figure legends, and β-galactosidase activity was measured as described previously (17).

Quantitative reverse transcription-PCR (RT-qPCR) was also used to examine *raiR* expression to include possible read-through transcription from the upstream *raiI* gene. The primers for *raiR* transcripts ($raiR_{RT-1}$ and $raiR_{RT-2}$) (see Table S1 in the supplemental material) were within the coding sequences of the *raiR* deletion construct. Wild-type and *raiR* mutants were grown to the late exponential phase (optical density at 600 nm [OD_{600}] of 0.8, and RNA was purified using an RNA extraction kit (TaKaRa). The cDNA was then synthesized using 5× All-In-One RT MasterMix (Applied Biological Materials), and real-time PCR was performed with SYBR Premix *Ex Taq* (Applied Biological Materials, Inc.) using a StepOne real-time PCR system (Eppendorf Realplex2). The quantity of cDNA measured by using a real-time PCR system was normalized to the abundance of 16S cDNA.

CinR purification and EMSA. The full-length CinR open reading frame (ORF) was amplified and cloned into pET28a(+). Recombinant CinR-His_6 was purified by Ni^{2+}-loaded nitrilotriacetic acid (NTA) resin (GE Healthcare) from cultures of *E. coli* BL21(DE3) carrying the resulting plasmid, according to the manufacturer's protocol. A biotin-labeled fragment containing the predicted *cinI* promoter region was PCR amplified with 5′ end biotin-labeled primers. CinI-producing AHLs were extracted from the cell-free supernatant of an *E. coli* strain expressing CinI or an *R. etli* triple AHL synthase mutant containing P_{lac}-*cinI*. The supernatants were extracted with dichloromethane, evaporated to dryness, and redissolved in a 1/1,000 volume of distilled H_2O (dH_2O). When indicated, 1 μl

of prepared AHL solution was included in the electrophoretic mobility shift assay (EMSA) reaction mix. Binding reaction mixtures contained 1 nM DNA and the amounts of CinR-His_6 indicated in Fig. 4 with or without AHLs in a buffer containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol (DTT), 60 mM potassium chloride, and 10% glycerol. After 30 min of incubation at 25°C, samples were size fractionated with the use of 5% polyacrylamide gels in 0.5× Tris-borate-EDTA (TBE) buffer. The band shifts were detected and analyzed by using a Chemiluminescent Nucleic Acid Detection Module kit (Thermo) according to the manufacturer's instructions. The images were obtained using an ImageQuant LAS 4000 Mini camera system (GE, Piscataway, NJ).

Plant assays. Common bean seeds were treated with 75% ethanol for 1 min and subsequently with 1.6% NaClO for 1 min. The treated seeds were then placed in sterilized culture dishes, germinated in the dark at 28°C for 2 days, and then planted in sterilized pots (five seeds per dish) filled with autoclaved vermiculite and a plant nutrient solution of Fahraeus medium (20) supplemented with 28 mg N/kg of vermiculite. Simultaneously, the autoclaved vermiculite was inoculated with approximately 10^8 *R. etli* cells (either wild type or mutant) cultured in PY medium. Plants were grown in a plant growth room at 28°C with 60% relative humidity and a 16-h–8-h day-night cycle. At the time indicated in Table 1, the plants were pulled out to count the number of nodules or to measure nitrogen fixing activity. There were at least 16 replicates per strain per experiment. *In vivo* nitrogenase activity was measured using an acetylene reduction assay according to previously described protocols (21, 22). In the absence of N_2, the enzyme catalyzes the conversion of acetylene (C_2H_2) to ethylene (C_2H_4) gas. Briefly, all nodules were collected from each plant, placed in 20-ml empty bottles with 2 ml of acetylene (10% vol/vol), and incubated upside down at 28°C for 2 h. The ethylene production was detected by using an HP 7280A series gas chromatograph system. Gas chromatography was conducted to measure peak height of ethylene and acetylene with 100 μl of gas. The approximate nitrogenase activity was expressed as μmol of C_2H_4 of acetylene production per gram of nodule dry weight.

RESULTS AND DISCUSSION

***R. etli* CFN42 possesses at least three LuxRI regulatory systems with different structures of AHLs.** The *R. etli* CFN42 genome contains three different LuxI-type AHL synthases (CinI, RaiI, and TraI) and four cognate LuxR-type regulators (CinR, RaiR, TraR1, and TraR2) (10) separately located on the chromosome and plasmids p42f and p42a (Fig. 1A). In addition to the previously reported TraI-TraR1-TraR2 system (12), sequence analysis demonstrated that putative CinI and CinR in *R. etli* also show a high degree of similarity at the amino acid level (95% and 97%, respectively) to CinI (GenBank accession number YP_768958.1) and CinR (YP_768957.1) from *R. leguminosarum* biovar viciae (AAF89900). Putative RaiI from *R. etli* shares 37% identity to RaiI (GenBank accession number CAK08866.1) from *R. leguminosarum* biovar viciae. The *raiR* gene, located 162 bp downstream of *raiI*, encodes a 212-amino-acid protein similar to BisR (GenBank accession number YP_768957.1) (31% identity) of *R. leguminosarum* biovar viciae.

To determine whether these LuxI family proteins are responsible for AHL synthesis in the *R. etli* CFN42 streptomycin-resistant derivative CE3, we first visualized AHL production in cell-free culture supernatant collected from the early stationary phase of the wild type and the triple LuxI deletion mutant Δ*cinI* Δ*raiI* Δ*traI* strain by applying a thin-layer chromatography (TLC) assay (16). As shown in Fig. 1B, high AHL activity was produced in wild-type cells, but no AHL was detected in the Δ*cinI* Δ*raiI* Δ*traI* mutants, indicating that in *R. etli*, CinI, RaiI, and TraI are responsible for all AHLs synthesized, at least under the growth condition tested. To determine the contents of AHL production of annotated AHL synthases, we expressed the *raiI*, *traI*, and *cinI* genes from a P_{tac} promoter carried on the low-copy-number plasmid pYC12 (23) in Δ*cinI* Δ*raiI* Δ*traI* mutants. TLC analysis shows that these three AHL synthases produced distinct AHLs in *R. etli* (Fig. 1B). Of note, expression of *raiI* and *traI* in the triple AHL synthase mutant produced additional spots in our TLC assays (Fig. 1B), possibly due to the overexpression of these AHL synthase genes. The structures of these AHLs produced in *R. etli* are under investigation.

***R. etli* QS deletion mutants alter AHL production.** To further investigate the functions of the *R. etli* QS regulatory genes, we first examined AHL production and AHL content changes in a series of in-frame mutations of three pairs of LuxRI homologues. Wild-type *R. etli* produced AHL molecules in a typical cell density-dependent manner, with low AHL activity at early time points and maximal levels in early-stationary-phase cultures (Fig. 2A and B, filled circles). Mutations in *raiI* and *raiR* did not significantly alter AHL production (Fig. 2A and B, filled squares). To ensure that this phenotype was not due to excessive input of supernatant into the bioassay strain, a series of dilutions for cell-free culture supernatants were assayed, and no significant difference in AHL production levels of these strains was observed (data not shown). Intriguingly, both *raiI* and *raiR* mutants produced more AHLs than the wild type in the TLC analysis (Fig. 2C) but not in a more quantitative liquid assay (Fig. 2A and B). The reason for this is not clear. Deletion of the TraI-TraR circuit, on the other hand, altered AHL production in *R. etli*, particularly during the early log phase (Fig. 2A and B, filled triangles). Complementation of *traI* or *traR* on a plasmid restored AHL production of the *traI* or *traR* mutant to the wild-type level (see Fig. S1A and B in the supplemental material), suggesting that the TraI-TraR system may be functional as early as early log phase. Mutation of *cinI* or *cinR* resulted in a dramatic decrease in AHL accumulation (Fig. 2A and B) as well as AHL content (Fig. 2C). Complementation of *cinI* or *cinR* on a plasmid in a *cinI* or *cinR* mutant restored AHL production (see Fig. S1A and B). In addition, AHL production was significantly reduced in all QS double and triple mutants tested (Fig. 2A and B, open symbols). These data imply that CinRI may be situated at the top of a cascade of other QS regulators.

CinR is required for *cinI* expression, and CinI-produced AHLs enhance CinR activity. To understand the possible interactions between different QS systems, we constructed QS regulatory gene promoter-*lacZ* fusion plasmids and examined β-galactosidase activity in the wild type and QS mutants. Compared to the wild type, *cinI* expression was abrogated in *cinR* mutants (Fig. 3A), indicating that CinR is required for *cinI* activation. Interestingly, *cinI* expression was reduced in *cinI* mutants as well as in triple AHL synthase mutants but not as severely as in *cinR* mutants (Fig. 3A), suggesting that CinR may be functionally independent of cognate AHLs. RaiRI and TraRI did not regulate *cinI* as *cinI* expression was unaltered in these mutants (Fig. 3A). To examine *cinI* expression in response to exogenous AHLs, we added AHLs produced by CinI, RaiI, or TraI to *cinI* mutants. We found that the expression of *cinI* was enhanced only in the presence of its cognate AHLs (Fig. 3B). We also examined the expression of *cinR*. We found that *cinR* expression was not regulated by other QS components, including CinR itself (Fig. 3C). These data suggest that CinR is required for *cinI* expression and that CinR may be active in the absence of AHLs.

To further investigate how CinR acts on the *cinI* promoter, we

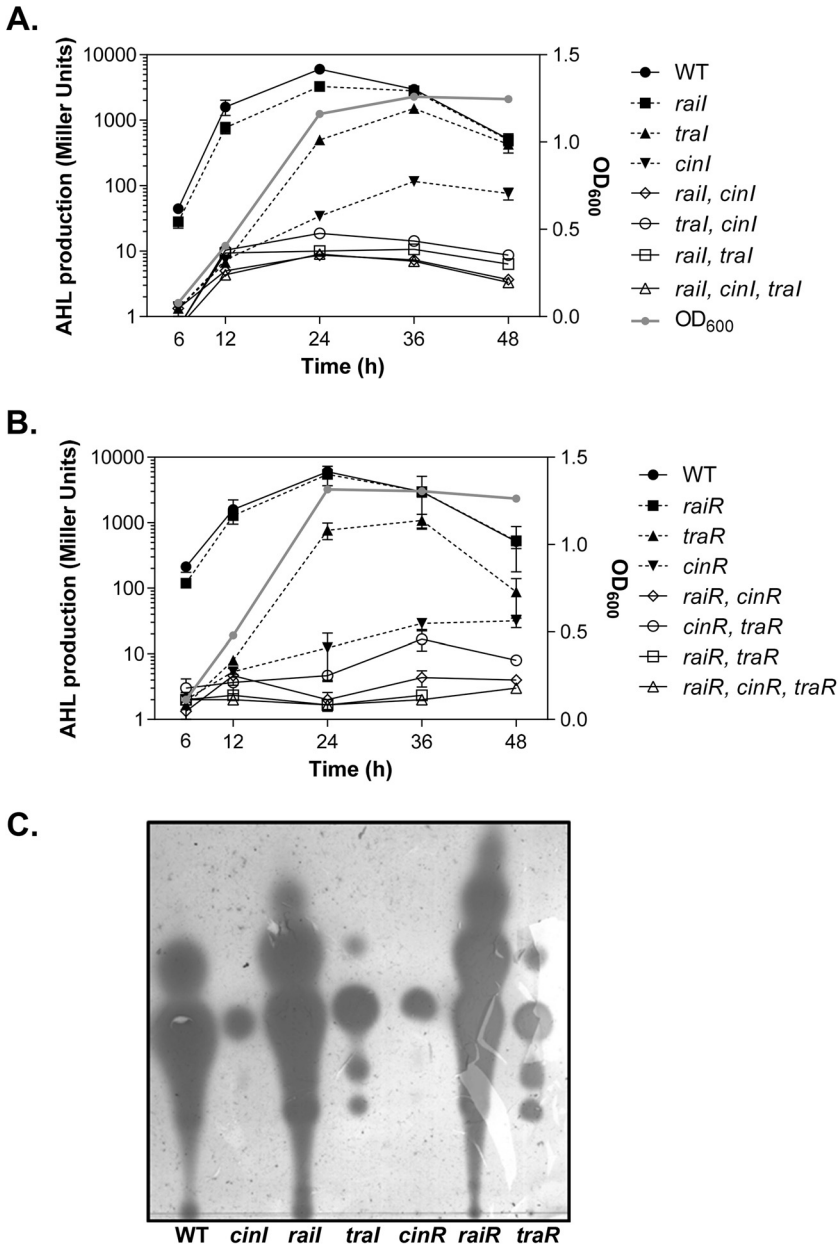

FIG 2 AHL production in *R. etli* QS mutants. Graphs represent the time course of accumulation of AHLs in the spent medium from LuxI-type QS mutants (A) and LuxR-type QS mutants (B). Bacterial cultures were withdrawn at the time points indicated to measure cell density (OD_{600}; gray lines) and AHL activity (black lines). *traR* represents the *traR1-traR2* double mutant. The data shown represent the means of three independent experiments, the standard deviations for which are indicated by the error bars. (C) TLC analysis of AHL contents. The wild-type and QS mutant cultures were extracted and loaded on a C_{18} reverse-phase TLC plate, followed by an overlay of the agar medium seeded with AHL bioassay strains. Each lane was loaded with 1 ml of culture extract. *traR* represents the *traR1-traR2* double mutant.

constructed and purified His-tagged CinR fusion recombinant proteins. We found that, unlike many other LuxR family proteins (24), CinR was soluble in the absence of its cognate AHLs (data not shown). This allowed us to evaluate DNA-binding affinity in the absence and presence of AHLs by electrophoretic mobility shift assays (EMSAs) using a biotin-labeled 300-bp DNA fragment containing the *cinI* promoter. The CinR protein displayed a specific affinity for the *cinI* promoter fragment in the absence of AHLs (Fig. 4A, top panel). Addition of unlabeled specific DNA abolished the shift, whereas adding unlabeled nonspecific competitive DNA did not alter the shift (Fig. 4A, top panel, last two lanes), indicating that the binding of CinR to the promoter is specific. Addition of cognate AHL (purified from cultures of *E. coli* that overexpressed *cinI*) in the binding reaction mixture caused CinR to form DNA-protein complexes at lower protein concentrations (Fig. 4A, lower panel). We estimate that the DNA-binding affinity of CinR to the *cinI* promoter in the presence of AHLs was approximately 20-fold higher than that of CinR in the absence of the signals (Fig. 4B). Additionally, we used AHLs purified from an *R. etli* triple AHL synthase mutant containing P_{lac}-*cinI* and found

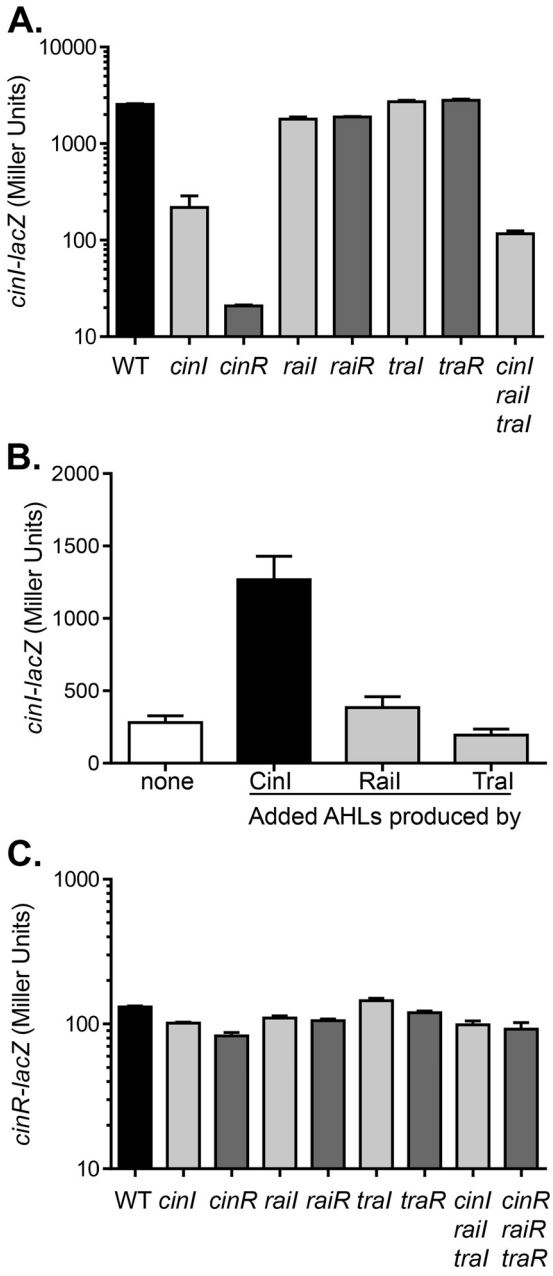

FIG 3 Roles of QS genes in *cinRI* expression. (A) The wild type and QS mutants containing *cinI-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. (B) *cinI-lacZ* expression in *cinI* mutants grown in PY medium supplemented with 5% cell-free supernatants from Δ*cinI* Δ*raiI* Δ*traI* mutants containing P_{tac}-*cinI*, P_{tac}-*raiI*, or P_{tac}-*traI* for 24 h. β-Galactosidase levels were then measured and reported in Miller units. (C) The wild type and QS mutants containing *cinR-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. The amount of β-galactosidase of a wild type containing the pRA302 vector control under the same growth condition was <10 units. The data shown represent the means of three independent experiments, the standard deviations for which are indicated by the error bars.

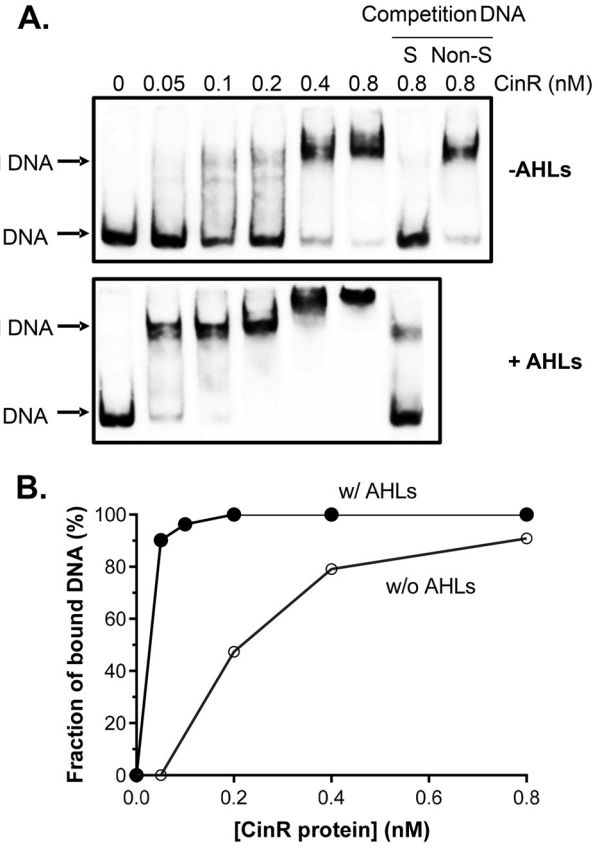

FIG 4 CinR protein binds to the *cinI* promoter DNA. (A) EMSA of purified recombinant CinR on *cinI* promoter DNA. Each lane contained 1 nM biotin-labeled *cinI* promoter DNA and the indicated concentration of CinR-His_6 protein in the absence (top panel) or presence (bottom panel) of AHLs produced by CinI expressed in *E. coli*. A 30-fold excess of either unlabeled *cinI* promoter DNA (S, specific) or random DNA fragments (Non-S, nonspecific) was used as competition DNA. (B) DNA binding affinity of CinR-His_6 in the absence (w/o) and the presence (w/) of AHLs. Results obtained from the experiment described in panel A were quantified by plotting the fraction of shifted specific DNA versus CinR-His_6 concentration.

that these AHLs could also enhance CinR binding of the *cinI* promoter, similar to those purified from *E. coli* (see Fig. S2 in the supplemental material). These results suggest that apo-CinR is able to bind its target promoter and that in the presence of its cognate ligands, CinR-AHL complex may bind to the target with higher affinity. The EMSA data are consistent with the *cinI-lacZ* expression profiles (Fig. 3A).

CinRI activates both RaiRI and TraRI circuits. To test whether the CinRI QS system affects the expression of the other two QS circuits, we examined the expression of *raiRI* and *traRI* in the wild-type and QS mutant strains. As shown in Fig. 5A, mutation of *raiR* completely abolished induction of *raiI*, but *raiI* itself did not significantly affect *raiI* expression. Addition of exogenous AHLs made by RaiI did not significantly enhance *raiI* expression (Fig. 5B). Mutation of *raiR* or *raiI* had almost no detectable effect on *raiR* expression (Fig. 5C). We also examined chromosomal *raiR* expression using RT-qPCR to verify if there are additional promoters upstream of *raiR* (e.g., from *raiI*). We found that *raiR* expression was not altered in the wild type or in the *raiR* mutants (see Fig. S2 in the supplemental

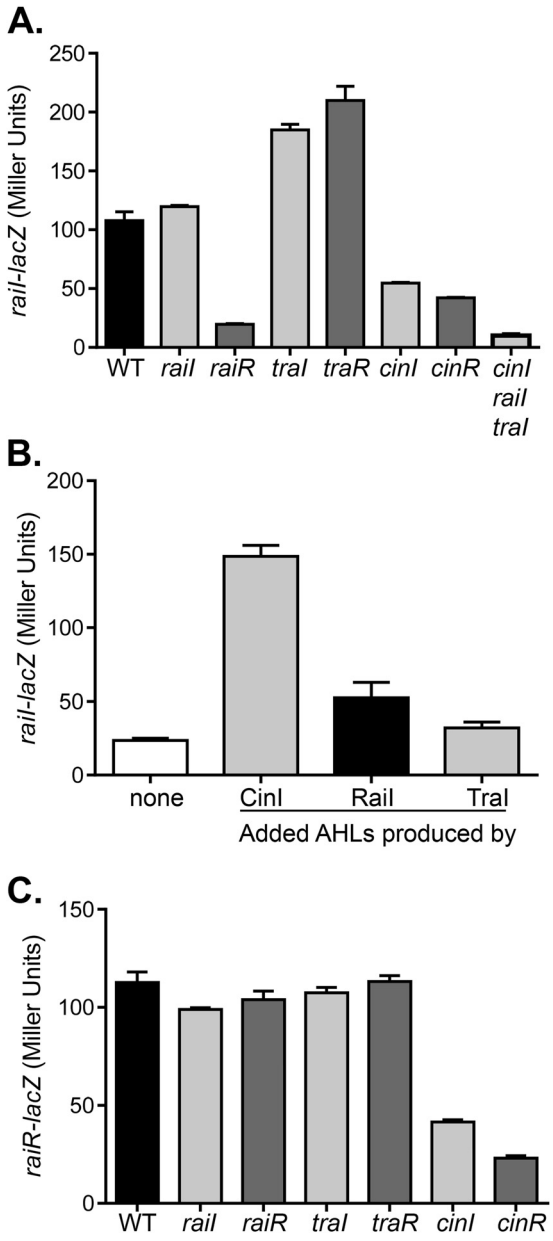

FIG 5 Roles of QS genes in *raiRI* expression. (A) The wild type and QS mutants containing *raiI-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. (B) *raiI-lacZ* expression in *raiI* mutants grown in PY medium supplemented with 5% cell-free supernatants from Δ*cinI* Δ*raiI* Δ*traI* mutants containing P_{tac}-*cinI*, P_{tac}-*raiI*, or P_{tac}-*traI* for 24 h. β-Galactosidase levels were then measured and are reported in Miller units. (C) The wild type and QS mutants containing *raiR-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. The amount of β-galactosidase of a wild type containing the vector pRA302 control under the same growth condition was <10 units. The data shown represent the means of three independent experiments, the standard deviations for which are indicated by the error bars.

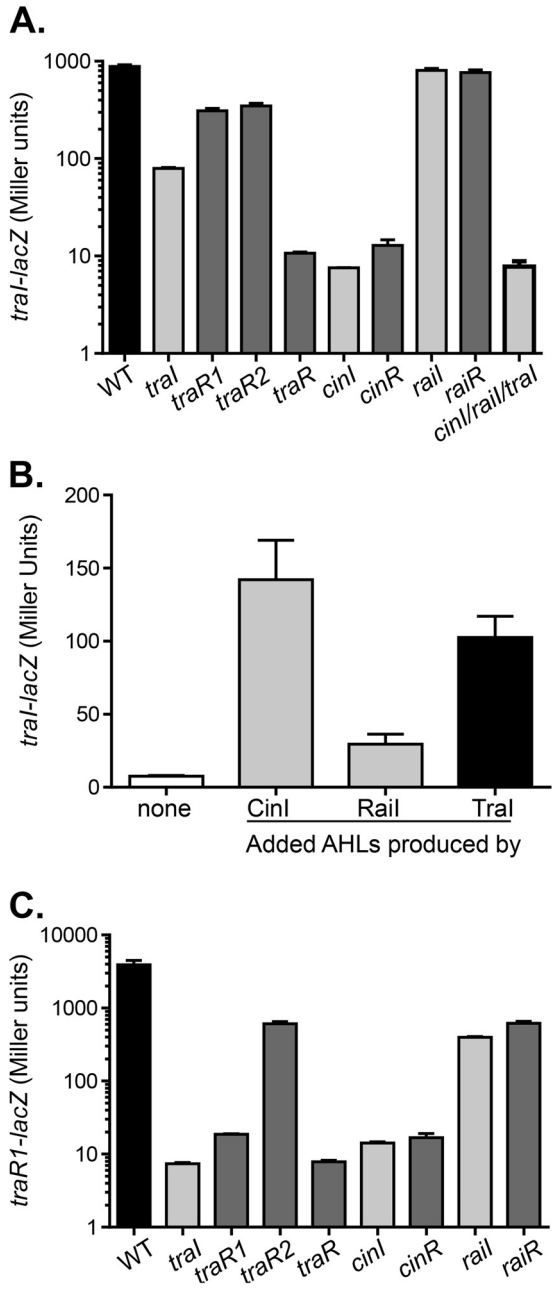

FIG 6 Roles of QS genes in *traRI* expression. (A) The wild type and QS mutants containing *traI-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. (B) *traI-lacZ* expression in *traI* mutants grown in PY medium supplemented with 5% cell-free supernatants from Δ*cinI* Δ*raiI* Δ*traI* mutants containing P_{tac}-*cinI*, P_{tac}-*raiI*, or P_{tac}-*traI* for 24 h. β-Galactosidase levels were then measured and are reported in Miller units. (C) The wild type and QS mutants containing *traR1-lacZ* plasmids were grown in PY medium for 24 h. Gene expression was analyzed by measuring β-galactosidase levels and is reported in Miller units. *traR* represents the *traR1-traR2* double mutant. The amount of β-galactosidase of a wild type containing the pRA302 vector control under the same growth condition was <10 units. The data shown represent the means of three independent experiments, the standard deviations for which are indicated by the error bars.

material). Taken together, these results suggest that *raiI* expression is independent of the cognate AHLs but dependent on RaiR, the expression of which is not autoregulated. Moreover, although the TraRI system had little, if any, effect on *raiRI*

TABLE 1 Symbiosis phenotypes of wild-type and QS mutant strains[a]

Strain[b]	First appearance of nodules (day)	Dry wt (mg/nodule)	Total no. of nodules	Pink nodule formation (%)[c]	Acetylene reduction (μmol/g nodules)
Wild type	7.3 (1.0)	59.6 (38.2)	93.3 (34.0)	89.5	20.4 (12.1)
cinR strain	7.4 (0.4)	48.3(33.1)	60.0 (20.7)**	100*	8.1 (4.3) **
cinI strain	8.0 (1.5)	56.3 (24.9)	103.9 (35.4)	84.8	15.0 (10.1)
raiR strain	7.1 (0.8)	45.4 (14.8)	73.9 (25.7)	82.5	11.4 (6.1) *
raiI strain	8.5 (0.3)	52.6 (17.4)	106.1 (49.8)	74.7*	17.1 (6.1)
traR strain	7.4 (0.6)	47.6 (18.1)	72.9 (31.3)	83.7	10.5 (9.9)
traI strain	6.8 (0.7)	51.7 (17.0)	80.4 (19.3)	87.2	7.8 (4.6) **

[a] Values are means (standard deviations) based on the examination of at least 16 replicates per strain. *, $P < 0.05$; **, $P < 0.005$ (Student *t* test).
[b] Blank inoculum did not form any nodules.
[c] Percentage of the total number of nodules.

expression, CinRI was apparently required for the full induction of *raiRI* (Fig. 5A and C). Additional AHLs made by CinI also stimulated *raiI* expression (Fig. 5B), which is again suggestive of the induction of *raiI* by CinRI.

We then examined *traRI* expression. We found that the level of *traI* expression was reduced by approximately 10-fold compared to that in the wild type. In the alignment of the *tra* locus, there are two adjacent LuxR-type QS regulatory genes, *traR1* and *traR2*, both of which had been reported to be required for inducing *traI* expression (12). As shown in Fig. 6A, each strain carrying a mutation in either of the two *traR* homologues resulted in about a 2.5-fold decrease in *traI* expression. Similarly, addition of exogenous AHLs produced by TraI to *traI* mutants restored *traI* expression (Fig. 6B). Moreover, *traI* expression was completely abolished in the *traR1-traR2* double mutants, suggesting that both TraR1 and TraR2 are involved in *traI* expression and that they function in parallel, similar to the regulation of the *tra* system in *R. leguminosarum* biovar viciae (25). Since *traR1* and *traR2* are likely to form an operon (www.microbesonline.org) (26), we studied only *traR1* expression. As shown in Fig. 6C, we found that the *traR1* mutation by itself, but not mutation of *traR2*, greatly reduced the expression of *traR1*, and the combination of mutations in *traR1* and *traR2* further abolished its expression. Furthermore, the *traI* mutation abrogated *traR1* expression. These results suggest that *traR1* expression is autoregulated in a cognate AHL-dependent fashion. In terms of cross talk regulation, RaiRI did not affect *traI* expression but had a slight impact on *traR1*, whereas CinRI was critical for the expression of both *traI* and *traR1* (Fig. 6A and C), and addition of AHLs produced by CinI stimulated *traI* expression (Fig. 6B), suggesting that CinRI is at the top of the QS regulatory network.

QS regulatory systems in *R. etli* CFN42 are important for symbiosis. Previous works have shown that QS is involved in symbiotic processes in the *R. etli* CNPAF512 strain (8, 9). However, it is not known how *R. etli* CFN42 QS contributes to symbiosis. We thus examined wild-type *R. etli* CFN42 and its QS mutants for their ability to nodulate and fix nitrogen on the common bean (*Phaseolus vulgaris* Linn). Although in all mutants nodules started forming at the same time as in the wild type and had an average size similar to that of the wild type, *cinR* mutants had reduced numbers of nodules formed (Table 1). Moreover, the N_2 fixation efficiency of nodules formed by *cinR*, *raiR*, and *traI* mutants was significantly lower than that of the wild type. Intriguingly, unlike *traI* mutants, *traR* mutants did not display a N_2 fixation deficiency. It is possible that an additional regulator is involved in TraI-produced AHL signals in nodules. Taken together, these data suggest that all three QS regulatory systems are involved in nodule formation and nitrogen fixing in *R. etli* CFN42. How QS regulates these symbiosis processes is under investigation.

QS systems in rhizobia have been shown to be complex and play important roles in symbiosis processes (2). In this study, we have identified three pairs of QS regulatory systems in *R. etli* CFN42: CinRI, RailRI, and TraRI. We found that CinRI regulates the other two circuits, which are also autoregulated (the working model is shown in Fig. 7). This QS regulatory pathway bears similarity to that in *R. leguminosarum* biovar viciae, which contains four different LuxRI-type QS systems, identified as *rai*, *tra*, *rhi*, and *cin* loci. As with *R. etli*, the *cin* locus is situated at the top of the hierarchical regulatory network and induces the other three QS systems (25, 27). CinR induces *cinI* expression in response to its signal, 3-OH-$C_{14:1}$-HSL. Mutation of *cinI* or *cinR* causes decreased production of all other short-chain AHLs synthesized by RaiI (28), TraI (25), and RhiI (29). RaiR induces *raiI* in response to RaiI-made AHLs and other noncognate AHLs, but mutations in *raiI* and *raiR* have no observable phenotypic effects on symbiosis (28). Both TraI-made and CinI-made AHLs induce the expression of *traR*, the gene product of which is necessary for symbiotic plasmid transfer (25). RhiR strongly induces *rhiI* in response to RhiI-made AHLs, and it is hypothesized that the *rhi* operon may play a role in the early stages of the symbiotic process (30). Although CinR-CinI is at the top of the regulatory cascade,

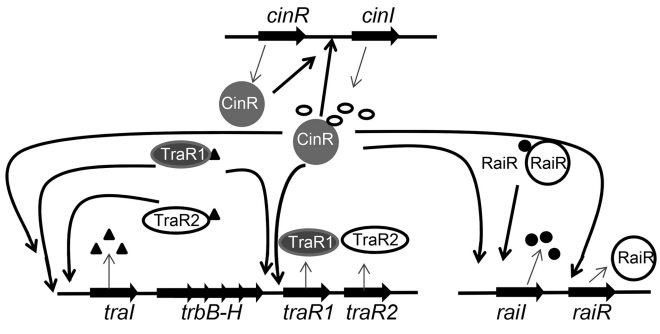

FIG 7 Model of the QS regulation network in *R. etli*. *R. etli* harbors three QS systems. The CinRI system activates CinR-mediated regulation of *cinI* to form a positive-feedback loop. CinRI also positively controls the *rai* and *tra* systems. *raiI* is activated by RaiR. In the *tra* system, TraR1 and TraR2 positively influence *traI* expression, which activates *traI* itself.

the mechanisms of regulation of *tra*, *rai*, and *rhi* loci are different. The *cin* genes activate the *tra* QS regulon via yet another LuxR-type regulator, BisR, the gene of which is located directly upstream of *traR* (25). CinR regulation of *rai* and *rhi* is coordinated by the *cinS* gene, which encodes a small regulatory protein cotranscribed with *cinI* (31). Additionally, the LuxR-type regulator ExpR is also required for full induction of the *raiR* gene (31).

Interestingly, CinR proteins in *R. etli* are soluble in the absence of their cognate ligands and can bind to *cinI* promoter DNA independent of AHLs (Fig. 4). Many other LuxR-type proteins, such as TraR (24), LasR (32), and LuxR (33), form inclusion bodies when overexpressed in *E. coli* in the absence of their cognate AHLs, whereas some LuxR-type proteins fold independent of their ligand, including MrtR in *Mesorhizobium tianshanense* (34), EsaR in *Pantoea stewartii* (35), ExpR in *Erwinia chrysanthemi* (36), and $CarR_{39006}$ in *Serratia* (37). However, these LuxR family proteins either require cognate AHLs (MrtR), are antagonized by their cognate AHLs (EsaR and ExoR) or are unaffected by AHLs (ligand-independent $CarR_{39006}$). In contrast, the CinR protein in *R. etli* is already active in the absence of AHLs and further activated by the cognate AHLs, a pattern never observed before and thereby representing a novel LuxR family protein. It is not clear whether this regulation has any physiological significance for *R. etli* biology and symbiosis. Further investigation is needed to fully understand how these three QS regulatory systems regulate *R. etli* symbiosis in natural environments.

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