JB
Journals.ASM.org

Identification and Characterization of a Second Quorum-Sensing System in *Agrobacterium tumefaciens* A6

Chao Wang,[a] Chunlan Yan,[b] Clay Fuqua,[c] Lian-Hui Zhang[a,d]

Institute of Molecular and Cell Biology, Singapore[a]; College of Life Science, South-Central University for Nationalities, Wuhan, China[b]; Department of Biology, Indiana University, Bloomington, Indiana, USA[c]; Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou, China[d]

Quorum sensing (QS) is a widespread mechanism of bacterial communication in which individual cells produce and respond to small chemical signals. In *Agrobacterium tumefaciens*, an acylhomoserine lactone-dependent QS mechanism is known to regulate the replication and conjugation of the tumor-inducing (Ti) plasmid. Most of the QS regulatory proteins are encoded within the Ti plasmid. Among them, TraI is the LuxI-type enzyme synthesizing the QS signal *N*-3-oxooctanoyl-L-homoserine lactone (3OC8HSL), TraR is the LuxR-type transcriptional factor that recognizes 3OC8HSL, and TraM is an antiactivator that antagonizes TraR. Recently, we identified a TraM homolog encoded by the *traM2* gene in the chromosomal background of *A. tumefaciens* A6. In this study, we further identified additional homologs (TraI2 and TraR2) of TraI and TraR in this strain. We showed that similar to TraI, TraI2 could predominantly synthesize the QS signal 3OC8HSL. We also showed that TraR2 could recognize 3OC8HSL and activate the *tra* box-containing promoters as efficiently as TraR. Further analysis showed that *traM2*, *traI2*, and *traR2* are physically linked on a mobile genetic element that is not related to the Ti plasmid. These findings indicate that *A. tumefaciens* A6 carries a second QS system that may play a redundant role in the regulation of the replication and conjugation of the Ti plasmid.

Quorum sensing (QS) is a bacterial community genetic regulatory mechanism that controls diverse biological functions in different bacterial species. Among the various bacterial QS systems reported, the best-characterized one is the acylhomoserine lactone (AHL)-based QS system. In this system, the LuxI (I)- and LuxR (R)-type proteins appear to be the central components. The I-type protein is the AHL synthase, and the R-type protein is the AHL-responsive transcription factor; they are conserved in different bacterial species containing AHL-based QS systems (1). In most cases, at a low bacterial population density, the I-type enzyme produces a basal level of AHL signals that accumulate as bacterial cells proliferate and interact with a cognate R-type transcription factor. Subsequently, the R-AHL complexes induce higher-level expression of I-type enzymes, which boosts AHL production and activates the transcriptional expression of other QS-dependent genes (2). It is through QS that individual bacterial cells could behave as a coordinated community in performing various biological activities, such as the production of secondary metabolites, the synthesis of virulence factors, and the development of biofilms.

Agrobacterium tumefaciens has been extensively studied as a model pathogen for the investigation of microbe-host interactions. Upon recognizing the chemical signals produced by host plants, *A. tumefaciens* infects a variety of plants and causes the crown gall diseases, which result in substantial losses of agricultural production worldwide. During infection, a DNA fragment (T-DNA) is transferred from the bacterial cells into the host plant cells and integrated into the chromosomal DNA (3–5). T-DNA, together with the genes associated with its interkingdom gene transfer, is located on the Ti plasmid (2). Interestingly, many environmental *Agrobacterium* isolates do not harbor the Ti plasmid and hence are avirulent. Therefore, conjugative transfer of the Ti plasmid from pathogenic strains to plasmid-free strains could play a key role in maintaining and expanding the population of infectious *A. tumefaciens* (6–8). In *A. tumefaciens*, the key QS regulators are encoded by the Ti plasmid, which includes TraI and TraR. TraI synthesizes the AHL signal *N*-3-oxooctanoyl-L-homoserine lactone (3OC8HSL), which binds to and activates TraR (9–11). TraR then binds to the palindromic *tra* box and thereby activates a number of operons that encode proteins necessary for Ti plasmid replication and conjugation (7, 12–14). In this context, the QS system of *A. tumefaciens* is similar to the prototype LuxI-LuxR QS system of *Vibrio* species.

However, the regulatory mechanisms of the *A. tumefaciens* QS system appear to be more complicated than the prototype mechanisms. First, this QS system is normally not active until bacterial cells detect the conjugative opines produced by the crown gall tumors incited by the pathogen (15). Furthermore, when the TraR level is low, TraM binds to TraR and forms an inactive complex that sets off the QS system until the cell density is high (16–18). TraM, functioning as a TraR antiactivator, has been identified in part of the agrobacterial species. Therefore, species-specific opines and TraM constitute additional regulatory components of the QS system of *A. tumefaciens* and ensure that Ti plasmid conjugation occurs only under certain conditions. In addition to this species specificity, the QS system of *A. tumefaciens* also displays strain-specific features. For example, in octopine-type strains, the conjugative opine for QS-dependent Ti plasmid conjugative transfer

Received 13 November 2013 **Accepted** 16 January 2014
Published ahead of print 24 January 2014
Address correspondence to Lian-Hui Zhang, lianhui@imcb.a-star.edu.sg.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.01351-13.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.01351-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
A. tumefaciens		
A6	Wild-type octopine strain	A. Kerr
A6(*traM*$_{k588}$)	A6 with *traM* replaced with *traM* of strain K588, which is dysfunctional	25
A6(*traM*$_{k588}$ Δ*traM2*)	A6(*traM*$_{k588}$) carrying in-frame deletion of *traM2*	25
A6(*traM*$_{k588}$ Δ*traM2*; *traR2*::Tn*5*)	A6(*traM*$_{k588}$ Δ*traM2*) carrying Tn*5* insertion of *traR2*, Kan^r	This study
A6(*traM*$_{k588}$ pDSK-*traR2*)	A6(*traM*$_{k588}$) with overexpression of *traR2*, Kan^r	This study
NT1(pDSK-*traR* pLA-*traG*::*lacZ*)	Also named NT1(*traR tra*::*lacZ749*), AHL signal reporter strain, Kan^r Tc^r	9
A6(*traM*$_{k588}$ pLA-*traG*::*lacZ*)	A6(*traM*$_{k588}$) carrying AHL-responsive reporter plasmid pLA-*traG*::*lacZ*, Tc^r	This study
A6(*traM*$_{k588}$ Δ*traM2* pLA-*traG*::*lacZ*)	A6(*traM*$_{k588}$ Δ*traM2*) carrying pLA-*traG*::*lacZ* vector, Tc^r	This study
A6(*traM*$_{k588}$ Δ*traM2 traR2*::Tn*5* pLA-*traG*::*lacZ*)	A6(*traM*$_{k588}$ Δ*traM2*; *traR2*::Tn*5*) carrying pLA-*traG*::*lacZ* vector, Kan^r Tc^r	This study
A6(*traM*$_{k588}$ pDSK-*traR2* pLA-*traG*::*lacZ*)	A6(*traM*$_{k588}$, pDSK-*traR2*) carrying pLA-*traG*::*lacZ* vector, Kan^r Tc^r	This study
C58C1	Derivative of nopaline strain C58 cured of Ti plasmid used for conjugation analysis, Rif^r	S. Q. Pan
Ach5C3	Derivative of wild-type octopine strain Ach5, Ti plasmid cured, Rif^r	S. K. Farrand
Ach5C3(pLA-*traG*::*lacZ*)	Ach5C3 carrying pLA-*traG*::*lacZ* vector, Tc^r	This study
Ach5C3(pLA-*traG*::*lacZ* pDSK-*traR2*)	Ach5C3(pLA-*traG*::*lacZ*) carrying pDSK-*traR2* vector, Kan^r Tc^r	This study
A6(*traM*$_{k588}$, Δ*traI*)	A6(*traM*$_{k588}$) with in-frame deletion of *traI* gene	This study
A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*)	A6(*traM*$_{k588}$, Δ*traI*) with Tn*5* insertion of *traI2*, Kna^r	This study
A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5* pLA-*traI2*)	A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*) carrying plasmid pLA-*traI2*, Kna^r Tc^r	This study
A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5* pLA-*traI*)	A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*) carrying plasmid pLA-*traI*, Kna^r Tc^r	This study
C1A6MR1	Transconjugant of A6(*traM*$_{k588}$ Δ*traM2*; *traR2*::Tn*5*) and C58C1, Rif^r Kan^r	This study
C1A6MR2	Transconjugant of A6(*traM*$_{k588}$ Δ*traM2*; *traR2*::Tn*5*) and C58C1, Rif^r Kan^r	This study
C1A6MR3	Transconjugant of A6(*traM*$_{k588}$ Δ*traM2*; *traR2*::Tn*5*) and C58C1, Rif^r Kan^r	This study
C58C1(pTiA6)	C58C1 carrying Ti plasmid of A6	25
E. coli		
DH5α (λpir)	*supE44* Δ*lacU169*(ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* λpir	Laboratory collection
BW020767(pRL27)	Harbors Tn*5* for *A. tumefaciens* mutagenesis	49
Plasmids		
pK18mobsacB	Broad-host-range gene replacement vector, Suc^r Kan^r	50
pK18-*traI*	pK18mobsacB harboring deleted *traI*-flanking region, Kan^r	This study
pLAFR3	IncP, broad-host-range cosmid vector, Tc^r	51
pLA-*traG*::*lacZ*	pLAR3 carrying *traG* gene transcriptionally fused with promoterless *lacZ*, Tc^r	9
pLA-*traI*	pLAFR3 harboring *traI* gene from A6, Tc^r	This study
pLA-*traI2*	pLAFR3 harboring *traI2* gene from A6, Tc^r	This study
pDSK	IncQ, broad-host-range plasmid, Kan^r	52
pDSK-*traR*	pDSK harboring *traR* gene from A6, Kan^r	9
pDSK-*traR2*	pDSK harboring *traR2* gene from A6, Kan^r	This study

[a] Abbreviations: Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Tc^r, tetracycline resistant; Rif^r, rifampincin resistant; Suc^r, sucrose resistant.

is octopine (19), while in nopaline-type strains, it is agrocinopines A and B (20). In addition, in nopaline strain C58, *traR* is a member of a five-gene operon of pTiC58, which is expressed from a promoter regulated by the transcriptional repressor AccR. Repression by AccR is relieved in the presence of agrocinopine A or B, and the operon, including *traR*, is expressed (19). In contrast, in octopine strains, *traR* is located in a 14-member operon that is regulated by the transcription factor OccR (21). OccR acts as either a repressor or an activator, depending on the absence or presence of octopine, respectively, by binding to different positions of the promoter of the *traR* operon (22). Furthermore, in octopine strains, the QS system is also negatively regulated by TrlR, a truncated version of TraR. TrlR antagonizes the activity of TraR by the formation of inactive heterodimers (23, 24). In contrast, no TrlR homolog is found in nopaline strains. Moreover, while TraM encoded by the Ti plasmid is a conserved QS modulator in both octopine- and nopaline-type strains of *A. tumefaciens*, a TraM homolog (TraM2) has recently been identified as an extra TraR antiactivator for QS regulation. Like TrlR, TraM2 exists in only some of the octopine strains (25). Put together, these findings have illustrated the complexity and genetic variations of the *A. tumefaciens* QS system.

In this investigation, we further studied the regulation of QS in *A. tumefaciens*. Using genetic approaches, we showed that *traR2* and *traI2* encode a second QS system (QS2) in *A. tumefaciens* strain A6. Bioinformatic and biochemical analyses showed that TraI2 is a functional homolog of TraI that predominantly synthesizes 3OC8HSL and that TraR2 is a functional homolog of TraR that recognizes 3OC8HSL and activates the QS-responsive genes. A conjugal transfer assay showed that *traR2* and *traI2*, together with the previously identified gene *traM2*, are located on a mobile genetic element. Phylogenetic analysis suggested that QS2 may represent an ancient paradigm of the QS systems of rhizobial species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* strains were grown at 28°C in LB medium (containing [per liter] 10 g Bacto

tryptone, 5 g yeast extract, and 10 g NaCl, pH 7.0) or in BM minimal medium (basic minimal-nutrient medium with 0.2% mannitol added as the sole carbon source and 0.2% ammonia sulfate as the sole nitrogen source unless otherwise indicated) (26). For bacterial growth assays, mannitol was replaced with octopine (0.2 g/liter). *Escherichia coli* strains were grown at 37°C in LB medium. Antibiotics were added at the following concentrations when required: kanamycin, 50 μg/ml (*A. tumefaciens*) or 100 μg/ml (*E. coli*); tetracycline, 5 μg/ml (*A. tumefaciens*) or 10 μg/ml (*E. coli*); rifampin, 100 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml.

DNA manipulation and plasmid construction. Plasmids were purified with the Plasmid Miniprep kit as recommended by the manufacturer (Qiagen). PCR product purification and DNA recovery from agarose gel were carried out with the QIAquick PCR Purification kit and the QIAquick Gel Extraction kit (Qiagen), respectively. For gene deletion construct pK18-*traI*, the DNA fragments flanking *traI* were separately amplified from *A. tumefaciens* strain A6 with two PCR primer pairs (5′-GCTCTAGAAGTGGGCGGGAACAGCG/5′-CGGGATCCTGGTGTTGGTATTGGTCGG and 5′-CGGGATCCGTTCGATTATCGCTGACG/5′-GCTCTAGATCTCGGCAATGATGGCATTG [enzyme cut sites are underlined]), digested with BamHI, and ligated with each other for PCR amplification with primers 5′-GCTCTAGAAGTGGGCGGGAACAGCG and 5′-GCTCTAGATCTCGGCAATGATGGCATTG. The PCR products were rescued from agarose gel and cut with XbaI for subsequent ligation with XbaI-digested vector pK18mobsacB. The resultant plasmid was screened by PCR and confirmed by DNA sequencing. For the preparation of expression constructs pLA-*traI* and pLA-*traI2*, *traI* and *traI2*, respectively, were amplified with PCR primer pairs 5′-CGGGATCCATGCTGATTCTGACCGTCTC/5′-CGGGATCCTCACGCCGCACTCCTCAAC and 5′-GGAATTCCATGCGGATCCTGACCGTTC/5′-GGAATTCACGCCGCGCTCCTCGCCG (enzyme cut sites are underlined), cut with BamHI and EcoRI, and then inserted into the pLAR3 vector by placing *traI* and *traI2* separately under the control of the *lac* promoter carried by the vector. For the construction of expression plasmid pDSK-*traR2*, the *traR2* gene was cloned from *A. tumefaciens* A6 with PCR primers 5′-ACCCACCTTACACATCAAGC/5′-CACAAGGTGCGGCATCGTAT, enzymatically digested, and linked into the vector pDSK by placing the gene under the control of the vector-borne *lac* promoter. pLA-*traG*::*lacZ* and pDSK-*traR* were isolated from *A. tumefaciens* NT1 (*traR tra*::*lacZ749*) (9).

Genetic manipulation of *A. tumefaciens*. Plasmids were transformed into *A. tumefaciens* by electroporation unless otherwise specified. The in-frame deletion of *traI* from strain A6($traM_{k588}$) was carried out as described previously (25). Conjugation analysis was performed on solid plates with octopine (0.2 g/liter) induction as reported previously (25). Tn*5* transposon mutagenesis of *A. tumefaciens* was performed as described previously (27). Ti plasmid conjugation transfer efficiency was examined by octopine induction on solid plates as described previously (25). Tumorigenicity assay of *A. tumefaciens* on plants was conducted as previously described (28).

Quantification of β-galactosidase activity. Quantitative analysis of β-galactosidase activity in bacterial cultures of *A. tumefaciens* strains was conducted as previously described (29). β-Galactosidase activity was measured and expressed in units per 10^9 CFU.

Detection of AHLs. The amount of AHL signals produced by bacterial cells was determined as described previously (30). The AHL indicator strain was *A. tumefaciens* NT1(*traR tra*::*lacZ749*), unless stated otherwise. Bacteria were grown overnight at 28°C for measurement of AHL production, and the amount of AHL production was calculated and expressed as the equivalent of 3OC8HSL as described previously (31, 32). Thin-layer chromatography (TLC) analysis of AHLs was carried out as reported previously (33). Briefly, bacterial strains were grown in 90 ml BM medium overnight and the supernatants were extracted with 200 ml ethyl acetate. The organic extracts were then condensed down to 180 μl in methanol. Two-microliter samples were then separated with a 50:50 methanol-water mixture on a C_{18} TLC plate with 1 μl (1 μM) standard 3OC8HSL. The signals were visualized by overlaying NT1(*traR lacZ749*) on the TLC plate.

RNA preparation and RT-PCR analysis. Bacterial strains were cultivated in minimal medium at 28°C with shaking at 200 rpm. When the optical density at 600 nm (OD_{600}) reached approximately 0.5, cells were collected and total RNA was isolated with the RNeasy Minikit (Qiagen). Residual DNA present in the RNA samples was removed by digestion with RNase-free DNase I. The quantity and quality of the RNA samples were examined with NanoDrop ND-1000 (NanoDrop Technologies) and by agarose gel electrophoresis. An aliquot of 0.2 μg of total RNA was serially 10-fold diluted and used as the template for one-step reverse transcription (RT)-PCR analysis (Qiagen). The PCR primer pairs used for RT-PCR analysis were as follows: *traM2*, 5′-GCGCGGACTTTCAAGCG/5′-AAACCCAGAAGTCCAACCAGC; *traR2*, 5′-GACTGCACAATACCCGGCC/5′-TCCGCTCCACGTAAACACTC; *traI2*, 5′-CCCACCTATGTCCTTGCG/5′-CGGTATTGTCAATCGCTACG; *traI*, 5′-CGACCAATACCAACACCAG/5′-GTTCGAAGCGAAGATCGG.

RESULTS

TraR2 is an additional regulator governing AHL production in *A. tumefaciens* strain A6. We showed previously that a single amino acid mutation (L54P) in the QS antiactivator TraM is responsible for the constitutive phenotype of *A. tumefaciens* K588 in QS; i.e., the strain could produce AHL signals and transfer the Ti plasmid in the absence of the cognate conjugal inducer octopine (25). K588 is a strain with a nopaline-type chromosomal background harboring an octopine-type Ti plasmid. To investigate the molecular basis of the QS-constitutive phenotype of *A. tumefaciens* K588, we introduced the L54P point mutation into TraM of *A. tumefaciens* wild-type strain A6 by allelic replacement to generate strain A6($traM_{k588}$). Because of the presence of *traM2* in the chromosomal background of A6, however, A6($traM_{k588}$) exhibited QS-related phenotypes indistinguishable from those of strain A6, including the production of AHL signals and the efficiency of Ti plasmid conjugal transfer (25). When cultured on an agar plate, A6($traM_{k588}$) could produce a detectable amount of AHLs, and deletion of the second copy of *traM* (*traM2*) significantly increased its AHL production (Fig. 1A), which is consistent with the previous findings (25). In this study, we used strain A6($traM_{k588}$ Δ*traM2*) to explore additional elements involved in QS regulation in *A. tumefaciens* by using A6($traM_{k588}$) as a control. Given that the AHL signals produced by A6($traM_{k588}$) in liquid medium without the addition of the inducer octopine were not detectable (25), solid agar plates were used to examine AHL production. Using A6($traM_{k588}$ Δ*traM2*) as the parent strain, we carried out Tn*5* transposon mutagenesis to screen for mutants with altered AHL production. The screening of >10,000 mutants identified 1 showing substantially decreased AHL production, which was comparable to that of A6($traM_{k588}$) (Fig. 1A). Analysis of the transposon-flanking sequences showed that the transposon was inserted into an open reading frame (ORF) that encodes a *traR* homolog, which was designated *traR2*.

To verify that the decrease in AHL production is due to the defect in *traR2*, we carried out a phenotypic analysis by the constitutive expression of *traR2* in the mutant A6($traM_{k588}$). As shown in Fig. 1A, the *traR2*-overexpressing strain had dramatically enhanced AHL production, comparable to that of A6($traM_{k588}$ Δ*traM2*), suggesting that TraR2 positively regulates the QS system in *A. tumefaciens* A6.

To further verify the involvement of *traR2* in AHL production in *A. tumefaciens* A6, we transferred the low-copy-number re-

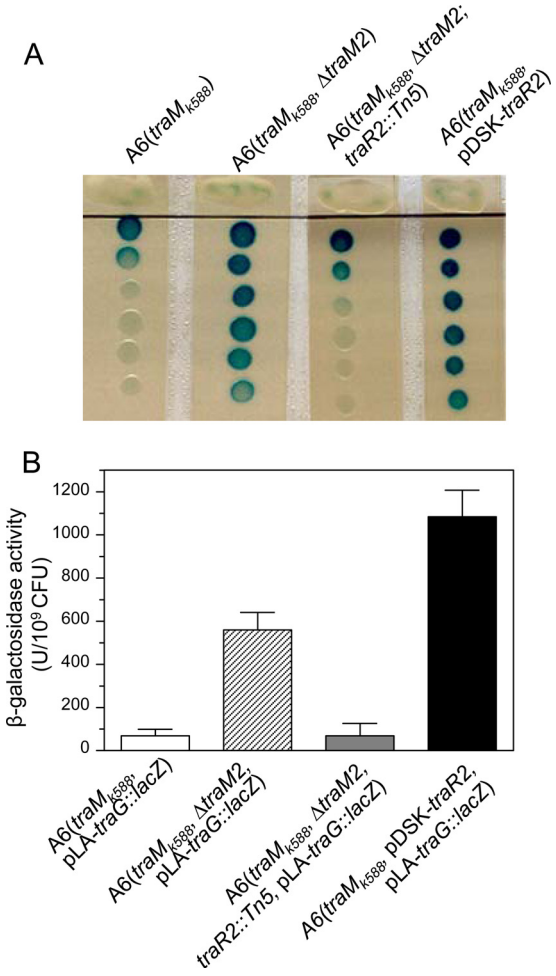

FIG 1 Characterization of the *traR2* gene associated with AHL production in *A. tumefaciens* A6. (A) AHL production by A6(*traM*$_{k588}$) and its derivatives on BM agar plates. (B) β-Galactosidase activity of A6(*traM*$_{k588}$ pLA-*traG*::*lacZ*) and its derivatives grown in liquid BM medium.

porter vector pLA-*traG*::*lacZ*, where the promoterless *lacZ* fragment was transcriptionally fused with the AHL-responsive promoter of the *traG* gene, into the *traR2*-deficient strains and then analyzed their AHL production as indicated by their β-galactosidase activity. The results showed that the β-galactosidase activity of A6(*traM*$_{k588}$ Δ*traM2*) was approximately 100 times that of A6(*traM*$_{k588}$). However, when *traR2* was mutated in the *traM* double mutant, its β-galactosidase activity was decreased considerably, to a level comparable to that of the *traM* single mutant (Fig. 1B). Moreover, overexpression of *traR2* in A6(*traM*$_{k588}$) dramatically enhanced its β-galactosidase activity to a level even higher than that of parent strain A6(*traM*$_{k588}$ Δ*traM2*) (Fig. 1B). These findings agree well with the AHL production results (Fig. 1A), supporting the notion that *traR2* functions as a positive regulator of QS in *A. tumefaciens* A6.

In silico analysis showed that *traR2* is about 67 and 68% identical to the *traR* gene of strain A6 at the nucleotide and peptide levels, respectively. The predicted secondary structures of TraR2 appear similar to those of TraR, containing 10 α-helixes and five β-sheets. Inspection of the N-terminal domain sequence of TraR2 showed that it is about 61% identical to the same domain of TraR, suggesting a high degree of conservation of AHL binding sites. Among the 13 amino acids important for ligand binding, 11 are identical between TraR and TraR2 (see Fig. S1B in the supplemental material).

Inspection of the C-terminal domain sequence also showed a high level of conservation between TraR2 and TraR of *A. tumefaciens* A6, whose amino acid sequences are about 71% identical. However, among the 10 key amino acids of TraR associated with DNA binding, TraR2 contains four varied residues (R206K, V207S, R210E, and M213R) (see Fig. S1B in the supplemental material). According to the X-ray crystal structure of TraR, the side chains of Arg206 and Arg210 make base-specific interactions, forming a hydrogen bond with the bases G and T in the Tra box, respectively, whereas the side chains of Val207 and Met213 form hydrophobic interactions with the backbone sugar of the bases G and T, respectively. Considering that all four of these residues are supposed to be determinants of the specific recognition by TraR of its cognate DNA target, alteration of these amino acids may indicate variations in the DNA recognition specificity and DNA binding affinity of these two QS regulators in *A. tumefaciens* A6.

The DNA sequences of the *traR2*-flanking segments were determined by the chromosome walking approach. The sequenced segment stretches a length of ~6 kb and encodes five putative ORFs. Downstream of *traR2*, three ORFs were identified that are homologs of *traM*, *traH*, and part of *traB* of the Ti plasmid, respectively, with identities ranging from 66 to 69% (see Fig. S1A in the supplemental material). Moreover, the genetic location and transcriptional orientation of these homologs appeared similar to those of their counterparts in the Ti plasmid, suggesting a highly conserved syntenic organization. However, no obvious ORFs could be defined within the ~3-kb region upstream of *traR2*, different from the case of the Ti plasmid, in which *ophED* is located immediately upstream of *traR* and constitutes a cotranscriptional operon with *traR* and upstream genes (see Fig. S1A in the supplemental material).

TraR2 is a functional 3OC8HSL receptor. For further investigation of the biological roles of *traR2* in *A. tumefaciens*, the *traR2* gene was heterogeneously and constitutively activated by the *lac* promoter of pDSK in Ach5C3, an octopine-type *A. tumefaciens* strain cured of the Ti plasmid and thus lacking the TraR-, TraM-, and TraI-encoding components of the QS system carried by the Ti plasmid. The AHL reporter plasmid of pLA-*traG*::*lacZ* was then introduced to monitor the response of TraR2 to external AHL signals. As shown in Fig. 2, when chemically synthesized 3OC8HSL was added at various concentrations, Ach5C3 and Ach5C3(pLA-*traG*::*lacZ*) both exhibited only background levels of β-galactosidase activity. When 3OC8HSL was added to Ach5C3(pDSK-*traR2* pLA-*traG*::*lacZ*), however, its β-galactosidase activity was substantially increased to a level comparable to that of control strain NT1(pDSK-*traR* pLA-*traG*::*lacZ*), in which the *traR* gene of the Ti plasmid was constitutively expressed by the *lac* promoter of vector pDSK. These findings demonstrate that TraR2 alone could also serve as an AHL receptor and activate the typical QS-dependent gene expression in *A. tumefaciens*. Additionally, it was also noticed that the β-galactosidase activity of Ach5C3(pDSK-*traR2* pLA-*traG*::*lacZ*) was much lower at low concentrations of 3OC8HSL but became relatively higher at high concentrations of 3OC8HSL than that of NT1(pDSK-*traR* pLA-*traG*::*lacZ*), respectively (Fig. 2), suggesting that the divergence in the DNA-binding domain may affect their promoter binding af-

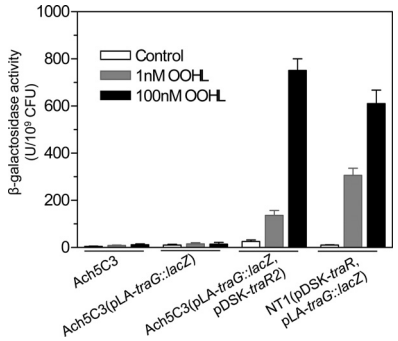

FIG 2 Functional analysis of TraR2. The β-galactosidase activities of Ach5C3 and its relevant derivatives were determined after incubation with various concentrations of 3OC8HSL signal molecules.

finity. These results may suggest that TraR and TraR2 could play different roles under various physiological conditions of AHL signals.

***A. tumefaciens* A6 contains a *traI* homolog *traI2*.** In species such as *Rhizobium leguminosarum*, several sets of AHL-type QS systems have been identified (36). The identification of second copies of *traM* and *traR* tempted us to assume that there may exist another copy of *traI* in *A. tumefaciens* A6. To test this assumption, we deleted the *traI* gene of the Ti plasmid from A6(*traM*$_{k588}$) and then examined its AHL production. Deletion of *traI* did not significantly affect AHL production on solid BM plates, indicating the existence of another copy of *traI* in *A. tumefaciens* A6 (see Fig. 4A). For identification of the *traI* homolog, A6(*traM*$_{k588}$ Δ*traI*) was used as the parent strain for Tn*5* transposon mutagenesis to screen for AHL-defective mutants. Among 5,000 transposon mutants screened, 1 was isolated that could not produce detectable AHLs on solid BM plates (Fig. 3). As could be expected, analysis of transposon-flanking sequences showed Tn*5* inserted within a *traI* homolog, which was thus named *traI2*. The *traI2* gene encodes a peptide of 212 amino acids with a deduced molecular mass of 23.6 kDa. Sequence alignment showed that the TraI2 peptide is about 69% identical to the peptide encoded by the *traI* gene of *A. tumefaciens* A6. Compared with TraI, both ends of TraI2 appeared highly variable while the central part was relatively conserved (see Fig. S2B in the supplemental material). Similar to the sequence downstream of *traI* of the Ti plasmid, the sequence downstream of *traI2* contains the homologs of *trbB*, *trbC*, *trbD*, and *trbE*, respectively, which likely form a cotranscriptional operon together with *traI*. However, no typical ORFs could be found upstream of

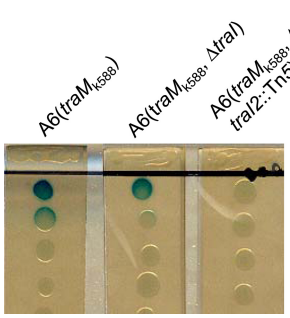

FIG 3 AHL production by A6(*traM*$_{k588}$) and its derivatives on BM agar plates.

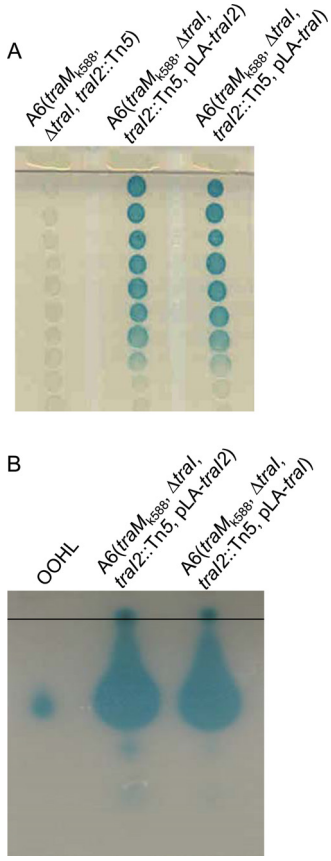

FIG 4 TraI2 is an AHL synthase. (A) AHL production by A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*) and its derivatives on BM agar plates. (B) TLC analysis of the AHL molecules produced by A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*) and its derivatives with chemically synthesized 3OC8HSL as the standard control.

the *traI2* gene, which was different from the corresponding region of the Ti plasmid, in which the *rep* operon containing *repA*, *repB*, and *repC* was divergently oriented in the region upstream of the *traI* gene (see Fig. S2A).

TraI2 is a 3OC8HSL synthase. To examine the biological function of TraI2 in *A. tumefaciens* A6, we overexpressed the *traI2* and *traI* genes, respectively, in the mutant A6(*traM*$_{k588}$ Δ*traI traI2*::Tn*5*) and determined its AHL production on solid BM plates. As shown in Fig. 4A, the *traI2*-overexpressing strain produced a large amount of AHLs, indistinguishable from that of the *traI*-overexpressing strain. This result demonstrates that TraI2 not only synthesized active AHLs but also synthesized the signals as efficiently as TraI in the background of octopine-type strain A6. Given that various active AHLs have been found in *A. tumefaciens* and the solid-plate-based bioassay system is not able to distinguish these signals, we used a TLC-based assay to analyze the AHLs produced by TraI2 and TraI in *A. tumefaciens* A6. The results showed that both TraI2 and TraI synthesized predominantly 3OC8HSL signals (Fig. 4B). These findings indicate that TraI2 is a functional 3OC8HSL synthase and thereby could participate in the regulation of Ti plasmid conjugal transfer.

QS2 is independent of octopine induction. It has been well established that the transcription of *traR* on the Ti plasmid is induced by octopine and controlled by QS itself in octopine-type *A. tumefaciens* strains. In the presence of octopine, the transcrip-

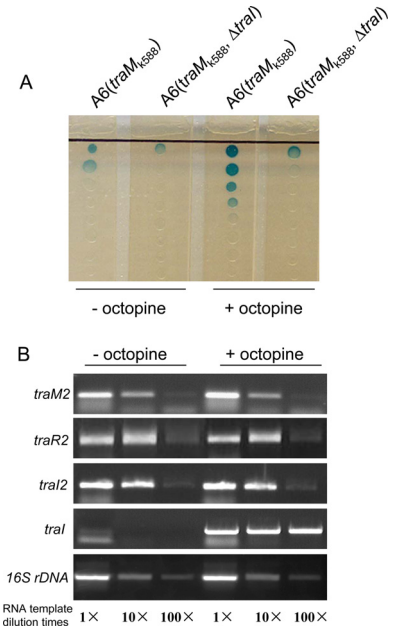

FIG 5 The QS2 system is independent of octopine induction in *A. tumefaciens* A6. (A) AHL production by A6(*traM*$_{k588}$) and A6(*traM*$_{k588}$ Δ*traI*) with (+) or without (−) octopine induction on BM agar plates. (B) RT-PCR analysis of *traM2*, *traR2*, and *traI2* expression with (+) or without (−) octopine induction with *traI* as a control. A6(pTiA6*traM*$_{K588}$) cells were cultivated in BM medium at 28°C with or without octopine induction. Samples were collected at OD_{600} of 1.2 for total RNA isolation. RT-PCR analysis was carried out with the primers described in Materials and Methods. Three concentrations of RNA templates, including an original concentration of 20 ng/μl and its 10× and 100× dilutions, were used for the comparative RT-PCR analysis.

tion of *traR* is activated and its product, TraR, forms a complex with the AHL signal 3OC8HSL. The TraR-AHL complex further increases the transcriptional expression of *traI*, *traM*, and *traR*. In this way, the QS regulatory mechanism forms a positive feedback loop triggered by octopine. The parallel roles of TraM2 and TraM, TraR2 and TraR, and TraI2 and TraI tempted us to examine the potential relationship of octopine induction and QS2 regulation. For this purpose, we first examined the AHL production of A6(*traM*$_{k588}$) and A6(*traM*$_{k588}$ Δ*traI*) with or without octopine induction. As shown in Fig. 5A, octopine treatment dramatically increased the AHL production of A6(*traM*$_{k588}$) but only marginally increased that of A6(*traM*$_{k588}$ Δ*traI*). These sharply contrasting results did not seem to be in favor of the notion that QS2, which includes TraM2, TraR2, and TraI2, could be induced by octopine. For further validation, RT-PCR was carried to study the transcription of the QS2-related genes in strain A6(*traM*$_{k588}$) with or without octopine. As a control, the transcription of *traI* was dramatically enhanced by octopine treatment; in contrast, however, the levels of *traM2*, *traR2*, and *traI2* transcripts did not show obvious changes regardless of the presence or absence of octopine (Fig. 5B). Taken together, these data demonstrate that the regulation of QS2 is independent of octopine induction, which is different from the regulatory mechanism of the Ti plasmid-borne QS system.

QS2 components are located on a transmissible genetic element. In *A. tumefaciens* strains, all of the QS regulatory genes are mapped on the Ti plasmid and thus these components are capable of transfer among the bacterial population via Ti plasmid conjugation. The tight linkage of *traM2*, *traR2*, and *traI2* with other homologs of the *tra* and *trb* genes suggested that the QS2 components may be located on a plasmid that is transmissible. To examine this hypothesis, we carried out the Ti plasmid conjugation assay with A6(*traM*$_{k588}$ Δ*traM2 traR2*::Tn*5*) as the donor strain and C58C1 as the recipient strain. The donor strain was resistant to kanamycin because the Tn*5* transposon was inserted into the *traR2* gene while the recipient strain was resistant to rifampin because of spontaneous mutation. After conjugation overnight with octopine induction, transconjugants were screened on LB agar plates supplied with kanamycin and rifampin. After 3 days of incubation at 28°C, single colonies appeared on the plates at a frequency of approximately 3.5×10^{-8}. Three transconjugants were randomly picked up for further phenotypic characterization. To test if *traI2* was transferred into these transconjugants, we detected their AHL production on the plate and found that all three of the transconjugants could produce detectable AHLs (Fig. 6A). PCR analysis showed that all of these colonies contained the *traI2* gene. As a control, the *ska* gene, which is present in C58C1 but absent from A6, could be successfully amplified from the transconjugants (Fig. 6B), supporting the idea that these colonies are nopaline-type C58C1 but not octopine-type A6 colonies. Additionally, the *traI2* and *traB2* segments, which are specific to the donor strain A6(*traM*$_{k588}$ Δ*traM2 traR2*::Tn*5*) but absent from the recipient strain C58C1, could also be amplified from these colonies, indicating that the transconjugants carried the *traI2*-containing fragment (Fig. 6B). Given that the *traB2* gene is physically linked with *traM2* and *traR2* (see Fig. S1A in the supplemental material), the above-described results also support the idea that the QS2 components (namely, *traM2*, *traR2*, and *traI2*) may reside on a single transmissible genetic unit. It was noted that the *traI* gene, which is unique to the donor strain, could also be amplified from one of the transconjugants, suggesting that this transconjugant also contains the Ti plasmid, which may account for the AHL overproduction phenotype of this transconjugant (Fig. 6A). Put together, these data demonstrate that the QS2 components are located on a transmissible genetic element, but its identity remains elusive. Using the transconjugant carrying this transmissible element alone, we tested the potential roles of this mobile genetic element. However, the results showed that this transmissible unit neither enabled the bacterial species to utilize octopine as its sole carbon source (Fig. 6C) nor incited crown gall tumor formation on a host plant (Fig. 6D).

Using A6(*traM*$_{k588}$ Δ*traM2 traR2*::Tn*5*) as the donor strain and C58C1 as the recipient strain, we also conducted a conjugation assay in the absence of octopine. However, no transconjugants were obtained on LB agar plates containing kanamycin and rifampin. These results suggest that octopine was essential for inducing the transmission of a QS2-containing genetic element. In *A. tumefaciens* A6, octopine has been known to trigger the expression of TraR, which could directly activate the *tra2* promoters, or it may activate only the *tra* promoters and subsequently the Tra proteins detect *oriC* for transmission of the QS2-containing genetic element. Further investigations are required to distinguish these two possibilities.

DISCUSSION

QS has been well established as a conserved regulatory mechanism for bacterial populations to coordinately regulate gene expression. The QS of *A. tumefaciens* could be one of the best-characterized

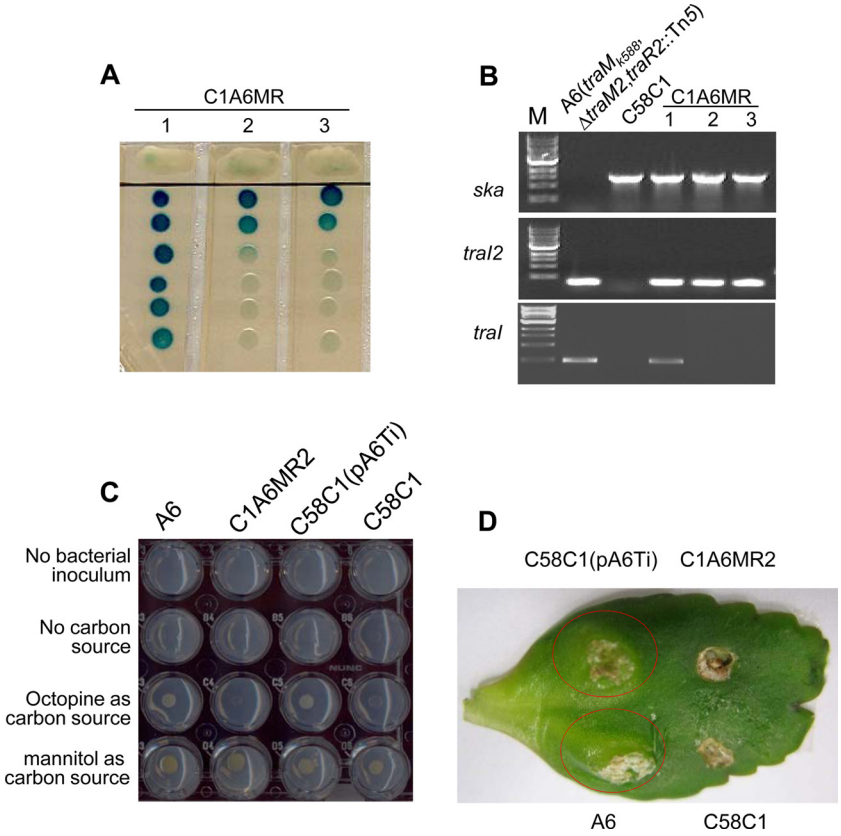

FIG 6 The QS2 genes are located on a transmissible genetic element in *A. tumefaciens* A6. (A) AHL production by three transconjugants (C1A6MR1 to C1A6MR3) obtained after conjugal mating with A6(*traM*$_{k588}$ Δ*traM2 traR2*::Tn*5*) as the donor strain and C58C1 as the recipient strain. (B) PCR examination of the donor, the recipient, and their transconjugants. The *ska* gene, which is unique to nopaline strain C58C1 and absent from octopine strain A6, and the *traI2* and *traI* fragments, which are present in A6 but absent from C58C1, were, respectively, amplified by PCR. Lanes M contained molecular size markers. (C) Bacterial growth of various *A. tumefaciens* strains with the carbon sources indicated. Results were recorded after 5 days of growth at 28°C. (D) Tumorigenicity assay of *A. tumefaciens* on kalanchoe leaves. Strains A6, C58C1, C1A6MR2, and C58C1(pTiA6) were grown in LB overnight and diluted to an OD_{600} of 1.0. Ten-microliter volumes of bacterial cultures were inoculated onto wounded kalanchoe leaves. The plant was grown at 24°C for 4 weeks before results were recorded, and the tumors incited by A6 and C58C1(pTiA6) are circled in red.

paradigms of QS systems. Several proteins, including TraI, TraR, and TraM, constitute the crucial components of QS regulation in this bacterium. TraI is an enzyme that synthesizes the signal molecule 3OC8HSL, which activates TraR. Activated TraR induces the transcriptional expression of its target genes, including the *tra*, *trb*, and *rep* operons. Moreover, the transcription of *traR* is initiated by cognate conjugal opines (37). In the octopine strains, placing *traI* within the QS-responsive *trb* operon constitutes a classic QS positive feedback loop found in many Gram-negative bacteria (38). In addition, a negative feedback loop is also formed by the TraM antiactivator, whose transcription is directly activated by the QS system (16). Previous data showed that these QS regulatory genes, including *traM*, are all located on the Ti plasmid. Recently, we identified TraM2 as an additional QS regulator that is not encoded by the Ti plasmid (25). TraM2 and its paralog TraM dually control Ti plasmid conjugation in *A. tumefaciens* A6. In this study, we continued to use the A6 strain to study the QS systems in *A. tumefaciens*, and we identified TraR2 and TraI2 as additional QS regulators in this bacterial species. TraR2, like TraR, recognizes 3OC8HSL and activates the QS-responsive reporter (Fig. 2). TraI2, similar to TraI, efficiently synthesizes 3OC8HSL in *A. tumefaciens* A6 (Fig. 4). Together with previously identified TraM2, TraR2 and TraI2 appear to constitute QS2 in *A. tumefaciens* A6. However, the regulation of QS2 is independent of octopine (Fig. 5), which differs from that of the Ti plasmid-borne QS system and highlights the complexity of QS regulation in *A. tumefaciens*. The next challenges would be to unravel the molecular mechanisms involved in QS2 regulation and to determine the extent to which these two QS systems could interact.

Identification of QS2 in this study also highlights the variations of QS systems in *A. tumefaciens*. Several previous lines of evidence have demonstrated the strain-specific variations of QS regulation in *A. tumefaciens*. First, host plants produce different opines to specifically activate the QS systems of different *A. tumefaciens* strains. For example, octopine and agrocinopine are the QS inducers of strains A6 and C58, respectively (20). Second, opine receptors may act differently in different strains. In A6, the receptor is OccR, which could act as an activator. In C58, however, the receptor is AccR, which functions only as a repressor. Third, the TrlR antagonist is absent from the C58 strain, while the TraM antiactivator is present only in octopine strains. Furthermore, TraM2, a TraM paralog initially identified in A6, was identified in only some of the octopine strains. The identification of TraR2 and TraI2 in A6 but not in other strains, such as C58, B6, K588, and

R10, adds another line of evidence for the strain specificity of the QS regulatory mechanisms in *A. tumefaciens*.

The identification of QS2 in *A. tumefaciens* is not the first example of a bacterial species with more than one AHL-type QS system. In *R. leguminosarum*, four QS systems have been identified and their roles are intertwined in a complex regulatory network regulating plasmid transfer, nodulation, bacterial growth, and other (unknown) functions (36, 37, 39, 40). In *R. etli* CNPAF512, two QS systems have been found that are involved in the regulation of symbiosome development and nitrogen fixation (41, 42). In these examples, each of the QS systems appeared to produce different AHL signal molecules and control different biological functions. In contrast, however, TraI2 of QS2 seemed to play a role identical to that of TraI of QS, with both synthesizing predominantly 3OC8HSL in *A. tumefaciens* A6. Moreover, TraR2 appeared to recognize 3OC8HSL and activate the *tra* box-containing promoters as efficiently as TraR. Put together, these results strongly suggest a signaling cross talk between QS and QS2 and we conclude that QS2 may play a role that is redundant with respect to that of QS in the regulation of Ti plasmid replication and conjugation. To date, however, we have not been able to rule out the possibility that QS2 is also involved in the regulation of other bacterial behaviors that are independent of QS control.

About 2 decades ago, we reported that octopine strains can be divided into two groups, transfer efficient (Tra^{e}) and transfer inefficient (Tra^{ie}), on the basis of their Ti plasmid conjugal transfer efficiency (26). The Tra^{e} group includes strains A6, NCPPB1001, and K608, whereas the Tra^{ie} group consists of B6S3, Ach5, and B6. It is interesting that Tra^{e} strain K608 is a conjugal transfer constitutive mutant (26) that is similar to strain K588, used in this study, which contains an L54P point mutation in TraM, and that QS2 genes were found in Tra^{e} strain A6 but not in Tra^{ie} strains B6S3, Ach5, and B6 (25). Therefore, the identification of the QS2 system in strain A6 in this study and the previous findings on the impact of the L54P mutation in TraM on QS (25) seem to provide two molecular mechanisms that could affect the conjugal transfer efficiency of the Ti plasmid in *A. tumefaciens*.

A cluster of genes in symbiotic plasmid pNGR234a of *Rhizobium* sp. strain NGR234 has been found to be highly homologous to the *tra* and QS system of *A. tumefaciens* (43, 44). Given the fact that the key QS regulators, i.e., TraI, TraR, and TraM, identified in this *Rhizobium* strain are the orthologs of *A. tumefaciens*, the QS of *Rhizobium* sp. strain NGR234 is proposed to regulate the transfer of pNGR234a (37). It has been demonstrated that the TraI homolog synthesizes 3OC8HSL in NGR234 (45). However, a *traI* mutant still produces an AHL signal that is likely to be 3OC8HSL, suggesting the presence of one or more additional AHL synthetases in this bacterial strain. Further analysis showed that a synthetase gene should reside elsewhere in the genome, but its identity has not yet been characterized (37). Our findings that TraI2 resides outside the Ti plasmid and predominantly produces 3OC8HSL in *A. tumefaciens* A6 may imply that strain NGR234 may also encode a TraI homolog, i.e., TraI2, that is responsible for the observed 3OC8HSL production of its *traI* mutant.

The results of this study show that the QS2 component genes (*traR2*, *traI2*, and *traM2*) are physically linked on a transmissible genetic unit. Given that their counterparts *traR*, *traI*, and *traM* are carried by the Ti plasmid, which is conjugally mobile (38), we speculated that the QS2 system genes could also be contained by a plasmid similar to the Ti plasmid. Using an approach described previously for Ti plasmid isolation (26), however, we failed to isolate the putative plasmid. One possible explanation for this failure is that this plasmid is too large to be isolated by routine methods, and the other possibility is that the QS2 system is carried not on a plasmid but in an integrative conjugative element, similar to the transmissible QS system in *Serratia marcescens* (46). It has been suggested that the multiple QS systems in most bacteria appear to have been acquired from separate sources (47, 48). Further characterization of the mobile genetic element that carries the QS2 genes may shade light on the origin of the QS2 system and the biological roles of this intriguing mechanism.

REFERENCES

1. **Fuqua C, Greenberg EP.** 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. **3:**685–695. http://dx.doi.org/10.1038/nrm907.
2. **Zhang LH.** 2003. Quorum quenching and proactive host defense. Trends Plant Sci. **8:**238–244. http://dx.doi.org/10.1016/S1360-1385(03)00063-3.
3. **Gheysen G, Montagu MV, Zambryski P.** 1987. Integration of Agrobacterium tumefaciens transfer DNA (T-DNA) involves rearrangements of target plant DNA sequences. Proc. Natl. Acad. Sci. U. S. A. **84:**6169–6173. http://dx.doi.org/10.1073/pnas.84.17.6169.
4. **McCullen CA, Binns AN.** 2006. Agrobacterium tumefaciens and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu. Rev. Cell Dev. Biol. **22:**101–127. http://dx.doi.org/10.1146/annurev.cellbio.22.011105.102022.
5. **Pitzschke A, Hirt H.** 2010. New insights into an old story: Agrobacterium-induced tumour formation in plants by plant transformation. EMBO J. **29:**1021–1032. http://dx.doi.org/10.1038/emboj.2010.8.
6. **Tsai CS, Winans SC.** 2010. LuxR-type quorum-sensing regulators that are detached from common scents. Mol. Microbiol. **77:**1072–1082. http://dx.doi.org/10.1111/j.1365-2958.2010.07279.x.
7. **Fuqua WC, Winans SC.** 1994. A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. **176:**2796–2806.
8. **Hwang I, Li PL, Zhang L, Piper KR, Cook DM, Tate ME, Farrand SK.** 1994. TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer. Proc. Natl. Acad. Sci. U. S. A. **91:**4639–4643. http://dx.doi.org/10.1073/pnas.91.11.4639.
9. **Piper KR, Beck von Bodman S, Farrand SK.** 1993. Conjugation factor of Agrobacterium tumefaciens regulates Ti plasmid transfer by autoinduction. Nature **362:**448–450. http://dx.doi.org/10.1038/362448a0.
10. **Zhang L, Murphy PJ, Kerr A, Tate ME.** 1993. Agrobacterium conjugation and gene regulation by *N*-acyl-L-homoserine lactones. Nature **362:**446–448. http://dx.doi.org/10.1038/362446a0.
11. **Qin Y, Luo ZQ, Smyth AJ, Gao P, Beck von Bodman S, Farrand SK.** 2000. Quorum-sensing signal binding results in dimerization of TraR and its release from membranes into the cytoplasm. EMBO J. **19:**5212–5221. http://dx.doi.org/10.1093/emboj/19.19.5212.
12. **Hwang I, Cook DM, Farrand SK.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J. Bacteriol. **177:**449–458.
13. **Pappas KM, Winans SC.** 2003. The RepA and RepB autorepressors and TraR play opposing roles in the regulation of a Ti plasmid repABC operon. Mol. Microbiol. **49:**441–455. http://dx.doi.org/10.1046/j.1365-2958.2003.03560.x.
14. **Pappas KM, Winans SC.** 2003. A LuxR-type regulator from Agrobacterium tumefaciens elevates Ti plasmid copy number by activating transcription of plasmid replication genes. Mol. Microbiol. **48:**1059–1073. http://dx.doi.org/10.1046/j.1365-2958.2003.03488.x.
15. **Farrand SK.** 1998. Conjugation in the Rhizobiaceae, p 199–233. *In* Spaink HP, Kondorosi A, Hooykaas PJJ (ed), The Rhizobiaceae: molecular biology of model plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
16. **Hwang I, Smyth AJ, Luo ZQ, Farrand SK.** 1999. Modulating quorum sensing by antiactivation: TraM interacts with TraR to inhibit activation of Ti plasmid conjugal transfer genes. Mol. Microbiol. **34:**282–294. http://dx.doi.org/10.1046/j.1365-2958.1999.01595.x.
17. **Luo ZQ, Qin Y, Farrand SK.** 2000. The antiactivator TraM interferes with

the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. J. Biol. Chem. **275:**7713–7722. http://dx.doi.org/10.1074/jbc.275.11.7713.
18. **Piper KR, Farrand SK.** 2000. Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. J. Bacteriol. **182:**1080–1088. http://dx.doi.org/10.1128/JB.182.4.1080-1088.2000.
19. **Petit A, Tempe J, Kerr A, Holsters M, Van Montagu M, Schell J.** 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature **271:**570–572. http://dx.doi.org/10.1038/271570a0.
20. **Ellis JG, Kerr A, Petit A, Tempe J.** 1982. Conjugal transfer of nopaline and agropine Ti-plasmids—the role of agrocinopines. Mol. Gen. Genet. **186:**269–274. http://dx.doi.org/10.1007/BF00331861.
21. **Fuqua C, Winans SC.** 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the traR gene of Ti plasmid pTiR10. Mol. Microbiol. **20:**1199–1210. http://dx.doi.org/10.1111/j.1365-2958.1996.tb02640.x.
22. **Wang L, Helmann JD, Winans SC.** 1992. The A. tumefaciens transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell **69:**659–667. http://dx.doi.org/10.1016/0092-8674(92)90229-6.
23. **Chai Y, Zhu J, Winans SC.** 2001. TrlR, a defective TraR-like protein of Agrobacterium tumefaciens, blocks TraR function in vitro by forming inactive TrlR:TraR dimers. Mol. Microbiol. **40:**414–421. http://dx.doi.org/10.1046/j.1365-2958.2001.02385.x.
24. **Oger P, Kim KS, Sackett RL, Piper KR, Farrand SK.** 1998. Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of traR, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. Mol. Microbiol. **27:**277–288. http://dx.doi.org/10.1046/j.1365-2958.1998.00671.x.
25. **Wang C, Zhang HB, Chen G, Chen L, Zhang LH.** 2006. Dual control of quorum sensing by two TraM-type antiactivators in *Agrobacterium tumefaciens* octopine strain A6. J. Bacteriol. **188:**2435–2445. http://dx.doi.org/10.1128/JB.188.7.2435-2445.2006.
26. **Zhang LH, Kerr A.** 1991. A diffusible compound can enhance conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens*. J. Bacteriol. **173:**1867–1872.
27. **Wang C, Zhang HB, Wang LH, Zhang LH.** 2006. Succinic semialdehyde couples stress response to quorum-sensing signal decay in Agrobacterium tumefaciens. Mol. Microbiol. **62:**45–56. http://dx.doi.org/10.1111/j.1365-2958.2006.05351.x.
28. **Xu XQ, Pan SQ.** 2000. An Agrobacterium catalase is a virulence factor involved in tumorigenesis. Mol. Microbiol. **35:**407–414. http://dx.doi.org/10.1046/j.1365-2958.2000.01709.x.
29. **Stachel SE, An G, Flores C, Nester EW.** 1985. A Tn3 lacZ transposon for the random generation of beta-galactosidase gene fusions: application to the analysis of gene expression in Agrobacterium. EMBO J. **4:**891–898.
30. **Dong YH, Xu JL, Li XZ, Zhang LH.** 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora. Proc. Natl. Acad. Sci. U. S. A. **97:**3526–3531. http://dx.doi.org/10.1073/pnas.97.7.3526.
31. **Zhang HB, Wang C, Zhang LH.** 2004. The quormone degradation system of Agrobacterium tumefaciens is regulated by starvation signal and stress alarmone (p) ppGpp. Mol. Microbiol. **52:**1389–1401. http://dx.doi.org/10.1111/j.1365-2958.2004.04061.x.
32. **Zhang HB, Wang LH, Zhang LH.** 2002. Genetic control of quorum-sensing signal turnover in Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. U. S. A. **99:**4638–4643. http://dx.doi.org/10.1073/pnas.022056699.
33. **Yang F, Wang LH, Wang J, Dong YH, Hu JY, Zhang LH.** 2005. Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. FEBS Lett. **579:**3713–3717. http://dx.doi.org/10.1016/j.febslet.2005.05.060.
34. Reference deleted.
35. Reference deleted.
36. **Rodelas B, Lithgow JK, Wisniewski-Dye F, Hardman A, Wilkinson A, Economou A, Williams P, Downie JA.** 1999. Analysis of quorum-sensing-dependent control of rhizosphere-expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. viciae. J. Bacteriol. **181:**3816–3823.
37. **González JE, Marketon MM.** 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol. Mol. Biol. Rev. **67:**574–592. http://dx.doi.org/10.1128/MMBR.67.4.574-592.2003.
38. **Zhu J, Oger PM, Schrammeijer B, Hooykaas PJ, Farrand SK, Winans SC.** 2000. The bases of crown gall tumorigenesis. J. Bacteriol. **182:**3885–3895. http://dx.doi.org/10.1128/JB.182.14.3885-3895.2000.
39. **Lithgow JK, Wilkinson A, Hardman A, Rodelas B, Wisniewski-Dye F, Williams P, Downie JA.** 2000. The regulatory locus cinRI in Rhizobium leguminosarum controls a network of quorum-sensing loci. Mol. Microbiol. **37:**81–97. http://dx.doi.org/10.1046/j.1365-2958.2000.01960.x.
40. **Wisniewski JP, Rathbun EA, Knox JP, Brewin NJ.** 2000. Involvement of diamine oxidase and peroxidase in insolubilization of the extracellular matrix: implications for pea nodule initiation by Rhizobium leguminosarum. Mol. Plant Microbe Interact. **13:**413–420. http://dx.doi.org/10.1094/MPMI.2000.13.4.413.
41. **Rosemeyer V, Michiels J, Verreth C, Vanderleyden J.** 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.
42. **Daniels R, De Vos DE, Desair J, Raedschelders G, Luyten E, Rosemeyer V, Verreth C, Schoeters E, Vanderleyden J, Michiels J.** 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J. Biol. Chem. **277:**462–468. http://dx.doi.org/10.1074/jbc.M106655200.
43. **Farrand SK, Hwang I, Cook DM.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J. Bacteriol. **178:**4233–4247.
44. **Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X.** 1997. Molecular basis of symbiosis between Rhizobium and legumes. Nature **387:**394–401. http://dx.doi.org/10.1038/387394a0.
45. **He X, Chang W, Pierce DL, Seib LO, Wagner J, Fuqua C.** 2003. Quorum sensing in *Rhizobium* sp. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. J. Bacteriol. **185:**809–822. http://dx.doi.org/10.1128/JB.185.3.809-822.2003.
46. **Wei JR, Tsai YH, Horng YT, Soo PC, Hsieh SC, Hsueh PR, Horng JT, Williams P, Lai HC.** 2006. A mobile quorum-sensing system in *Serratia marcescens*. J. Bacteriol. **188:**1518–1525. http://dx.doi.org/10.1128/JB.188.4.1518-1525.2006.
47. **Gray KM, Garey JR.** 2001. The evolution of bacterial LuxI and LuxR quorum sensing regulators. Microbiology **147:**2379–2387. http://mic.sgmjournals.org/content/147/8/2379.long.
48. **Lerat E, Moran NA.** 2004. The evolutionary history of quorum-sensing systems in bacteria. Mol. Biol. Evol. **21:**903–913. http://dx.doi.org/10.1093/molbev/msh097.
49. **Larsen RA, Wilson MM, Guss AM, Metcalf WW.** 2002. Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch. Microbiol. **178:**193–201. http://dx.doi.org/10.1007/s00203-002-0442-2.
50. **Simon R, Priefer U, Pühler A.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Nat. Biotechnol. **1:**784–791. http://dx.doi.org/10.1038/nbt1183-784.
51. **Staskawicz B, Dahlbeck D, Keen N, Napoli C.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
52. **Keen NT, Tamaki S, Kobayashi D, Trollinger D.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197. http://dx.doi.org/10.1016/0378-1119(88)90117-5.