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Dual Control of Quorum Sensing by Two TraM-Type Antiactivators in *Agrobacterium tumefaciens* Octopine Strain A6

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Agrobacterium tumefaciens **wild-type strains have a unique quorum-sensing (QS)-dependent Ti plasmid conjugative transfer phenotype in which QS signaling is activated by corresponding conjugative opine inducers. Strain K588, with a nopaline-type chromosomal background harboring an octopine-type Ti plasmid, however, is a spontaneous mutant displaying a constitutive phenotype in QS. In this study, we show that a single amino acid mutation (L54P) in the QS antiactivator TraM encoded by the *traM* gene of Ti plasmid is responsible for the constitutive phenotype of strain K588. Introduction of the L54P point mutation to the TraM of wild-type strain A6 by allelic replacement, however, failed to generate the expected constitutive phenotype in this octopine-type strain. Intriguingly, the QS-constitutive phenotype appeared when the pTiA6 carrying the mutated *traM* was placed in the chromosomal background of the nopaline-type strain C58C1RS, suggesting an unknown inhibitory factor(s) encoded by the chromosomal background of strain A6 but not by C58C1RS. Low-stringency Southern blotting analysis showed that strain A6, but not strain C58 and its derivatives, contains a second *traM* homologue. The homologue, designated *traM2*, has 64% and 65% identities with *traM* at the DNA and peptide levels, respectively. Similar to TraM, TraM2 is a potent antiactivator that functions by blocking TraR, the QS activator, from specific binding to the *tra* gene promoters. Deletion of *traM2* in strain A6 harboring the mutated *traM* confers a constitutive QS phenotype. The results demonstrate that the QS system in strain A6 is subjected to the dual control of TraM and TraM2.**

Quorum sensing (QS) is a bacterial community genetic regulatory mechanism that controls diverse biological functions in different bacterial species. Among the several bacterial QS systems reported (for reviews, see references 2, 32, and 38), the most characterized one is the acylhomoserine lactone (AHL)-based QS system. AHL signals are known to regulate many interesting microbial activities, including bioluminescence in *Vibrio* species (7), Ti plasmid conjugative transfer in *Agrobacterium tumefaciens* (23, 36), antibiotics production and virulence in *Erwinia carotovora* (25), and virulence and biofilm formation in *Pseudomonas aeruginosa* (5, 20). The central components of AHL-based QS systems are LuxI (I)- and LuxR (R)-type proteins. The former is the AHL synthase and the latter is the AHL-dependent transcription factor, both of which are conserved in different bacterial species containing AHL-based QS systems (11). In most cases, at low bacterial population density, the I-type enzyme produces a basal level of AHL signals, which accumulate as bacterial cells proliferate and interact with the R-type transcription factors. Subsequently, the R-AHL complexes induce higher-level expression of I-type enzymes, which boosts AHL production (AHL signals are also known as autoinducers), and activate the transcriptional expression of other QS-dependent genes (37).

Differing from the above generally conserved QS model, the QS system in the wild-type strains of *A. tumefaciens*, which regulates Ti plasmid conjugative transfer, is normally not active until the bacterial cells detect conjugative opines produced by the crown gall tumors incited by the *A. tumefaciens* strain harboring this Ti plasmid (9). The conjugative opines regulate QS and, hence, Ti plasmid conjugative transfer by controlling the expression of *traR*, which encodes the R-type protein of *A. tumefaciens*. More specifically, in *A. tumefaciens* octopine-type strains, the QS-dependent Ti plasmid conjugative transfer is induced by octopine (21), while in nopaline-type strains, it is induced by agrocinopines A and B (8). 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 (22), whereas in octopine strains, *traR* is located in a 14-member operon that is regulated by the transcription factor OccR (12). OccR acts either as 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 (31).

TraR activity is negatively modulated by antiactivator TraM. TraM interferes with TraR function by formation of a stable antiactivation complex that prevents TraR from recognizing its target DNA-binding sites (4, 17, 29, 30). It was shown that TraM is critical for setting the QS threshold in *A. tumefaciens* and for prevention of TraR from initiating transcription of the *tra* regulon in the absence of opine inducer (24). In octopine-mannityl opine-type strains, a Ti plasmid-encoded TrlR, which is a truncated version of TraR with the DNA-binding domain at its carboxyl terminus eliminated due to a frameshift mutation, could also interfere with the function of TraR (19, 41). Purified TrlR formed inactive heterodimers with TraR in the presence of AHL signals and blocked TraR for both specific DNA binding and transcription of a *tra* promoter (3).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotypes and/or characteristics[a]	Source or reference
A. tumefaciens		
NT1(*traR tra*::*lacZ749*)	C58C1RS harboring pJM749 and pSVB33, AHL bioassay indicator strain	25
K588	C58C1 harboring octopine type Ti plasmid pTiB6S3Tra^c, Ery^r, Chl^r	A. Kerr
A6	Wild-type octopine strain containing pTiA6, Tra^i	A. Kerr
Ach5	Wild-type octopine strain containing pTiAch5, Tra^i	C. Fuqua
R10	Wild-type octopine strain containing pTiR10, Tra^i	C. Fuqua
B6	Wild-type octopine strain containing pTiB6, Tra^i	C. Fuqua
B6S3	Wild-type octopine strain containing pTiB6S3, Tra^i	C. Fuqua
ATCC 15955	Wild-type octopine strain containing pTi15955, Tra^i	C. Fuqua
C58	Wild-type nopaline strain containing pTiC58, Tra^i	S. Q. Pan
C58C1RS	Derivative of strain C58, pTi$^-$, Rif^r, recipient strain for Ti plasmid conjugative transfer	S. Q. Pan
K749	Derivative of strain C58, pTi$^-$, pAT$^-$, Rif^r	A. Kerr
Ach5C3	Derivative of strain Ach5, Ti plasmid cured, Rif^r	S. K. Farrand
A6(pTiA6*traM*$_{K588}$)	Derivative of strain A6 where the *traM* gene was replaced with the *traM* from strain K588	This study
C58C1RS(pTiA6*traM*$_{K588}$)	Derivative of strain C58C1RS with the Ti plasmid donated by A6(pTiA6traM$_{K588}$)	This study
C58C1RS(pTiK588)	Derivative of strain C58C1RS harboring pTiK588 of K588	This study
Ach5C3(pTiA6*traM*$_{K588}$)	Derivative of strain Ach5C3 with the Ti plasmid donated by A6(pTiA6traM$_{K588}$)	This study
Ach5C3(pTiK588)	Derivative of strain Ach5C3 harboring pTiK588 of K588	This study
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* λ*pir*	Laboratory collection
S17-1	pro res$^-$ mod$^+$ integrated copy of RP4, mob$^+$	A. Kerr
Plasmids		
pLAFR3	IncP, broad-host-range cosmid vector, Tet^r	28
pAW19	f1(+)ori *lacZ*α of pBluescript II (SK+), *sacB*, Suc^r	S. K. Farrand
pLA-*traM*$_{A6}$-PT	pLAFR3 harboring the coding region of *traM* amplified from pTiA6; the gene is under the control of the *lac* promoter of the vector, Tet^r	This study
pLA-*traM2*	pLAFR3 harboring the coding region of *traM2* that is placed under the control of the *lac* promoter of the vector, Tet^r	This study
pJZ358	Expression construct for TraR$_{R10}$ expression	41
pGC-*traM2*	Expression construct for TraM2 expression	This study
pBK129	Cosmid clone containing about 26-kb *tra* region from pTiK588	This study
pAW19-*traM*$_{K588}$	pAW19 containing the *traM* coding sequences and its flanking region from pTiK588	This study
pAW-*traM2d*	pAW19 containing the flanking regions of *traM2* from strain A6	This study

[a] Abbreviations: Tra^c, constitutive in AHL production and Ti plasmid conjugative transfer; Tra^i, AHL production and Ti plasmid conjugative transfer are inducible by opine inducer; Amp^r, ampicilin resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant; Rif^r, rifampicin resistant; Ery^r, erythromycin resistant; Chl^r, chloramphenicol resistant; Suc^r, sucrose resistant.

In *A. tumefaciens*, production of the AHL signals, including *N*-(3-oxo-octanoyl)-L-homoserine lactone and other minor derivatives, is encoded by *traI* (15, 36), which is under the control of the transcription factor TraR. The findings that TraR is subject to several levels of regulations and modulations may explain our early observation that production of the AHL signal is hardly detectable in the wild-type *A. tumefaciens* strains in the absence of opine inducers (39). Nevertheless, spontaneous mutants of *A. tumefaciens*, which are constitutive in AHL signal production and Ti plasmid conjugative transfer, have been identified (1, 16, 39). Genetic analysis of a spontaneous Tra^c (transfer constitutive) mutant of nopaline strain C58 showed that mutation of the repressor AccR is responsible for the constitutive phenotype (1). Transposon mutagenesis studies revealed that knockout of *traM* can also lead to constitutive AHL production and Ti plasmid conjugative transfer in nopaline strain C58 and octopine strain R10 (10, 14).

We have previously reported a spontaneous Tra^c mutant K588 of *A. tumefaciens* (34); the mutant, carrying a Tra^c derivative of the octopine-type plasmid pTiB6S3 in the genetic background of nopaline-type strain C58C1RS, is constitutive in AHL production. Although the mechanism is unknown, the constitutive nature of this mutant has greatly facilitated the in vivo investigations of genetic regulation of QS signal degradation in *A. tumefaciens*, as a wild-type strain such as A6 does not produce a detectable level of AHL signals in liquid medium even in the presence of octopine (33, 34). In this study, we have investigated the QS constitutive nature of strain K588. By DNA sequencing and genetic analysis, we identified a single nucleotide mutation in the *traM* of pTiK588 that accounts for the QS constitutive phenotype of strain K588. Based on this finding, we questioned whether introduction of the same mutation in the *traM* of pTiA6 could also render a QS constitutive phenotype in strain A6. The analysis led to identification of a *traM* homologue in octopine-type strains A6 and Ach5C3. We further demonstrated that the *traM* homologue, designated *traM2*, also encodes a potent QS antiactivator.

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 (per liter contains 10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl, pH 7.0) or in BM minimal medium (basic minimum nutrient with 0.2% mannitol added as the sole carbon source and 0.2% ammonia

sulfate as the sole nitrogen source unless otherwise indicated) (33, 39). *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 using a 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 QIAquick gel extraction kit (QIAGEN), respectively. For construction of pAW-*traM*$_{K588}$, the DNA fragment containing *traM* was amplified from *A. tumefaciens* strain K588 with forward primer 5′-CCCAAGCTTGATCCTAACTATTTCTCCTG and reverse primer 5′-CGGGATCCGTTTCGAGGCTCATCAAAAG, end-blunted using Klenow enzyme before ligation to vector pAW19 (18), which was cut by NotI and subsequently filled in with Klenow enzyme. The resulting plasmid was screened by PCR and confirmed by DNA sequencing. The construct pLA-*traM*$_{A6}$–PT was assembled as follows: the allele containing the TraM-encoding region and its putative promoter was cloned from *A. tumefaciens* A6 with the PCR primers 5′-CCCAAGCTTGATCCTAACTATTTCTCCTG and 5′-CGGGATCCGTTTCGAGGCTCATCAAAAG (enzymatic cut sites underlined), enzyme digested, and introduced between the BamHI and HindIII sites of pLAFR3. The resulting construct was further analyzed to ensure that the insert was placed in the orientation opposite that of the *lac* promoter of the vector so that the *traM*$_{A6}$ expression was under the control of its cognate promoter.

Replacement of *traM*$_{A6}$ with *traM*$_{K588}$ in *A. tumefaciens* A6. The allelic replacement of *traM* in strain A6 (*traM*$_{A6}$) with that from strain K588 (*traM*$_{K588}$) was carried out following the procedure described previously (18) with minor modifications. Briefly, the plasmid pAW-*traM*$_{K588}$ was introduced into *E. coli* S17-1 by electroporation, and then kanamycin-resistant colonies of *E. coli* S17-1(pAW-*traM*$_{K588}$) were selected and mated with *A. tumefaciens* A6. The transconjugants were grown on BM medium supplemented with 50 μg/ml kanamycin for 4 days; a single colony was then picked and streaked for further purification. Subsequently, allelic replacement candidates were selected on BM agar plates containing 5% sucrose at 28°C (13). These candidates were then selected for loss of their kanamycin resistance on LB agar plates and confirmed by DNA sequencing.

Deletion of *traM2* in strain A6(pTiA6*traM*$_{K588}$) and complementation. Deletion of the *traM2* gene in strain A6(pTiA6*traM*$_{K588}$) was performed following the previously described method (18) with minor modifications. In brief, two PCR fragments flanking *traM2* were amplified from the genomic DNA sample of *A. tumefaciens* A6 with two pairs of PCR primers (5′-GAAGGTGTGGCGGTGTTC and 5′-CGGGATCCGACACTACTGAATCTTTC; 5′-CGGGATCCGCTGGTTGGACTTCTGG and 5′-CAGGAATCACTATCCCCA). The PCR products were digested with BamHI and ligated with T4 DNA ligase. The fusion fragment was amplified by PCR using ligation mixture as a template and a pair of primers (forward, 5′-GAAGGTGTGGCGGTGTTC; reverse, 5′-CAGGAATCACTATCCCCA). The product was purified using the QIAquick gel extraction kit (QIAGEN) after agarose gel separation. The fragment, filled in with Klenow enzyme, was cloned into the pAW19 vector which had been cut with NotI and blunted by Klenow enzyme; the resultant construct was designated pAW-*traM2d*. After DNA sequencing confirmation, pAW-*traM2d* was introduced into *E. coli* S17-1 by electroporation. The kanamycin-resistant colonies of *E. coli* S17-1(pAW-*traM2d*) were selected, confirmed by PCR, and mated with *A. tumefaciens* A6(pTiA6*traM*$_{K588}$) by biparental mating. Selection of kanamycin-resistant transconjugants and subsequent identification of the *traM2* deletion mutant were conducted as described above.

For complementation, the coding region of the native *traM2* gene was amplified with a primer pair (5′-CGGGATCCATGGATTTGAAAGATTCAG and 5′-CGGGATCCGCAGGCTTCAGTTGACC). The PCR products were digested with BamHI, purified with the QIAquick PCR purification kit (QIAGEN), and then ligated into the vector pLAFR3 cut with the same enzyme. The construct, in which *traM2* was placed under the control of the *lac* promoter from the vector, was selected after PCR and sequencing verification and introduced into *A. tumefaciens* strains by following the procedures described above.

Quantitative determination of AHL. The amount of AHL signals produced by bacterial cells was quantitatively determined as described previously (6). Briefly, 20 ml BM agar plates supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (50 μg/ml) were cut into separated slices (1 cm in width) and then used for AHL bioassay. To measure the concentration of AHL in bacterial supernatants, the cell culture was sampled at given time points and centrifuged at 14,000 rpm for 30 min at 4°C. Supernatants (5 μl) were added to one end of an agar slice, and AHL indicator strain NT1 (*traR tra*::*lacZ749*) was spotted at progressively further distances from the loaded samples. The plates were incubated at 28°C overnight, and the distance from the last induced blue colony to the origin of the AHL sample in each agar slice was measured for quantification of the amount of AHL, as described previously (6). To measure AHL production on solid agar plates, 10 μl of fresh culture with an optical density at 600 nm (OD_{600}) of 0.6 was spotted on bioassay plates and incubated at 28°C for 12 h before the indicator strain was loaded. Based on the signal diffusion distance, the amount of AHL produced was calculated and expressed as the equivalent of *N*-(3-oxo-octanoyl)-L-homoserine lactone, as described previously (6, 34). Each assay was repeated at least three times with two replicates.

Conjugative transfer efficiency assay. The Conjugation assay was carried out by modification of the method of drop mating described previously (39). The recipient strains used in this study were the rifampin-resistant *A. tumefaciens* nopaline strain C58C1RS and octopine strain Ach5C3 (Table 1). Donor strains were grown for 24 h at 28°C in BM minimal agar plates (when required, octopine was included at a final concentration of 0.8 g per liter). The bacterial cells were resuspended in BM medium without any carbon source and adjusted to an OD_{600} of 1.0, and a 10-fold dilution series was prepared. From each dilution, 5 μl of bacterial cells was spotted onto a lawn of the recipient strain growing on selective plates, which were supplemented with antibiotic rifampin and octopine as the only carbon source to support the growth of transconjugants only. To determine the number of viable donor cells, 10 μl of cells of each dilution was spread onto LB plates. Plates were incubated at 28°C. The numbers of donors and transconjugants were counted 1 week after mating. Each assay was repeated two times with three replicates. The transfer efficiency of the donor strain was calculated as the number of transconjugants observed per donor cell applied.

RNA preparation and real-time RT-PCR. Bacterial strains were cultivated in minimal medium with or without octopine, as indicated. When the OD_{600} reached 1.2, cells were collected by centrifugation at 4°C. Total RNA was subsequently isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Residual DNA present in the RNA samples was removed by the RNase-free DNase I. The quality and quantity of the RNA samples were determined with a Nanodrop ND-1000 (Nanodrop Technologies). An aliquot of 0.2 μg total RNA was used as a template for normal reverse transcription (RT)-PCR to amplify the *traI* coding region using forward primer 5′-CGACCAATACCAACACCAG-3′ and reverse primer 5′-GACGGTGTTGCCAATCGC-3′. A fragment of 16S rRNA was also amplified under the same conditions as the internal control using forward primer 5′-TGACGAGTGGCGGACGGGTG and reverse primer 5′-ATGCAGTTCCCAGGTTGAGC.

The real-time RT-PCR was conducted on a Lightcycler from Roche Diagnostics with the QuantiTect SYBR green RT-PCR kit (QIAGEN) according to the manufacturer's protocol. The *traI* cDNA fragments were amplified using primers 5′-CGACCAATACCAACACCAG-3′ and 5′-GACGGTGTTGCCAATCGC-3′. The 16S rRNA transcripts were included as an endogenous control for each sample using primers 5′-GAGGAAGGTGGGGATGACG and 5′-CCAACTCCCATGGTGTGACG. The data were analyzed using the LightCycler 3.5.3 software (Roche Diagnostics Corporation).

Southern blotting analysis and cloning of *traM2*. Total DNA samples were prepared from different strains using the MasterPure DNA purification kit (Epicenter Technologies, Madison, WI). Probes were generated by PCR from strain A6 with the PCR digoxigenin (DIG) labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations. Primers for *traM* and *traM2* amplifications were 5′-CGTGACGAAAAAGGTCGAG (forward) and 5′-GGGTATGAAGCCAAGAATG (reverse) and 5′-GATTCAGTAGTGTCGGAC (forward) and 5′-GATGAAACCCAGAAGTCC (reverse), respectively. Total DNA samples from different strains were completely digested by BamHI and separated on a 1.2% agarose gel, and Southern blotting analyses were performed with DIG-labeled probes according to the manufacturer's protocols (Roche Molecular Biochemicals, Mannheim, Germany). For blotting using *traM* probe, low-stringency hybridization was performed at 37°C and washed consecutively at 60°C twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer containing 0.1% sodium dodecyl sulfate (SDS) and twice with 0.5× SSC containing 0.1% SDS. For *traM2* blotting, high-stringency hybridization and washing were done at 42°C and 68°C, respectively, with the same initial washing buffer as above, but the washing buffer in the last two rounds was changed to 0.1× SSC containing 0.1% SDS. Signals were detected with the DIG luminescent detection kit (Roche Molecular Biochemicals, Mannheim, Germany).

For cloning of *traM2*, several pairs of PCR primers were designed based on the coding and flanking sequences of *traM* of strain A6. The primer pair that produced the expected DNA fragments was 5′-CGGATTCTAACCCCCGGC (forward) and 5′-TACCGCGCTCGCCATCCGG (reverse). The DNA fragments amplified from the total DNAs of *A. tumefaciens* strains were separated on 1.2% agarose gels and purified with the QIAquick gel extraction kit (QIAGEN) for DNA sequencing.

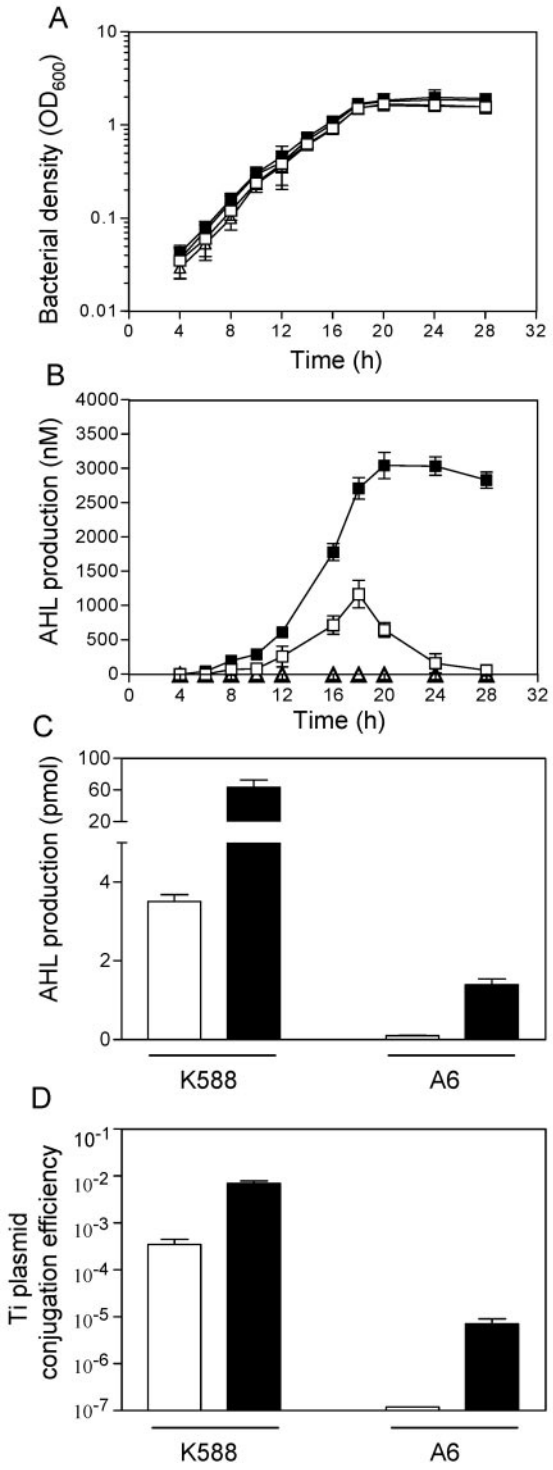

FIG. 1. Phenotype analysis of *A. tumefaciens* strains K588 (square) and A6 (triangle). The assays were performed with (solid symbol or bar) or without (open symbol or bar) octopine. (A) Time course of growth of the two strains in BM minimal medium. Bacterial overnight culture was diluted in BM medium to same population density (OD_{600} = 1.0), inoculated into BM medium at a 1:100 ratio, and incubated with shaking (200 rpm) at 28°C. (B) AHL production by *A. tumefaciens* strains in liquid cell culture. The culture conditions were the same as above. Note, the data for A6 with and without octopine were identical, and as a result, solid triangles overlapped with open triangles. (C) AHL production by *A. tumefaciens* strains on BM medium agar plate. The procedures for inoculation and bioassay were described in Materials and Methods. (D) Ti plasmid conjugative transfer efficiency of *A. tumefaciens* strains. Induction of donor cells was done on solid agar medium as described in Materials and Methods. Values are presented as means ± standard deviations of results from at least three independent repeats.

Gel mobility assay. The wild-type TraM and TraR, encoded by pTiR10, were purified as previously described (4). The coding region of *traM2* was cloned into pET15b (Novagen) at the NdeI and BamHI restriction sites, respectively, to generate plasmid pGC-*traM2*. TraM2 was expressed in *E. coli* BL21(DE3) harboring pGC-*traM2*. A gel mobility assay was performed as described before (29). An infrared dye (IR)-labeled *tra* box-containing DNA fragment was amplified from pJZ304 (42) by PCR using two synthetic oligonucleotides, 5′-AATTAAC CCTCACTAAAG (IRD700 labeled; LI-COR) and 5′-GTTTTCCCAGTCA CGAC (Integrated DNA Technologies). TraR (0.1 μM) was incubated with an IR-labeled *tra* box in reaction buffer (12 mM HEPES-NaOH, 4 mM Tris-HCl, 60 mM potassium glutamate, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol). TraM2 or TraM at the indicated concentrations was added to the above reaction mixture and incubated for 30 min at room temperature. The mixture was directly subject to 8% nondenaturing polyacrylamide (80:1 acrylamide:*bis*-acrylamide) gels. The IR signal was visualized in the Odyssey infrared image system (LI-COR).

RESULTS

Phenotype comparison of QS-constitutive strain K588 and QS-inducible strain A6. In an effort to elucidate the cause that rendered K588 the QS-constitutive phenotype, we made careful analyses of bacterial growth, AHL production, and Ti plasmid conjugative transfer efficiency by comparison with *A. tumefaciens* strain A6. A6 is a wild-type octopine-type strain carrying pTiA6, whose QS-dependent conjugative transfer is activated by induction of octopine (39). The results showed that K588 and A6 exhibited similar growth patterns; bacterial cells grew exponentially in minimal medium until about 18 h after inoculation, when they moved into stationary phase (Fig. 1A). Addition of octopine at the concentration of 0.8 mg/ml in BM minimal medium did not have a significant effect on bacterial growth. In the absence of octopine, AHL was detectable in the supernatant of K588 when the bacterial population density (OD_{600}) reached around 0.2. The signal molecules accumulated rapidly along with the exponential growth of the bacterial population and peaked at the transition to stationary phase, with a maximal concentration reaching about 1,200 nM, and then decreased quickly (Fig. 1B). In the presence of octopine, AHL could be detected in the K588 supernatant at an earlier stage (OD_{600} = ~0.1). Furthermore, the concentration was significantly higher than that without octopine induction throughout the growth phases, approximately threefold at the peak. The results suggest that even though the tight control of QS was released in K588, octopine could still significantly boost AHL accumulation. Strain A6, however, produced no detectable AHL in liquid culture even in the presence of octopine (Fig. 1B), indicating that its concentration in the supernatant was lower than 0.05 pmol, which is the detection threshold of the AHL reporter strain used in this study.

To provide more accurate comparison of the relative AHL production between the two strains, we measured the signal production by adding and growing same amount of K588 and A6 cells on solid BM agar plates. As shown in Fig. 1C, A6 produced a basal level of 0.15 ± 0.04 pmol when grown over-

night on octopine-free plates, whereas K588 produced about 3.5 ± 0.29 pmol, which was about 23-fold higher than A6. In the presence of octopine, the AHL production by A6 was increased up to 1.7 ± 0.8 pmol, showing a typical octopine-inducible phenotype. As expected, similar to what had been observed in liquid culture, K588 also produced substantially more AHL (60 ± 3.75 pmol) on plates containing octopine. It is noteworthy that the amount of AHL produced by A6 with octopine induction was still less than that of constitutively produced by K588, reflecting very strict regulatory mechanisms in strain A6.

Furthermore, we determined the Ti plasmid conjugative transfer efficiencies of strains K588 and A6 with and without octopine induction. The results (Fig. 1D) showed that the transfer efficiency of A6 was not detectable ($<10^{-9}$) in the absence of octopine but increased by at least 1,000-fold with octopine induction. In contrast, the QS-constitutive strain K588 transferred its Ti plasmid at a high frequency (4×10^{-4}), and octopine induction moderately increased the efficiency by about 10 times. Given the significant difference of the two strains in AHL production, we further tested whether the addition of AHL to strain A6 could bypass the requirement of octopine induction. However, no significant difference in Ti plasmid transfer efficiency of strain A6 was observed with or without external addition of the QS signals (1, 5, 10 μM). Taken together, these data suggest that the enhanced AHL production in strain K588 is not the primary cause responsible for the constitutive Ti plasmid conjugative transfer phenotype.

Sequence analysis of the key genes implicated in QS signal production and regulation in strains K588 and A6. It is known that the majority of the genes involved in QS signal production and Ti plasmid conjugative transfer are carried by Ti plasmid. To check if there was any significant insertion or deletion in pTiK588, we purified pTiK588 and pTiA6 from strains K588 and A6, respectively, using a previously established simple method (35). Digestion of the two plasmids with restriction enzymes EcoRI and HindIII, respectively, however, appeared to produce the same digestion pattern (data not shown) as that previously reported for pTiA6 (35), implying that point mutations or small deletions may be more likely the cause.

To identify the mutated gene(s) responsible for the QS- and Ti plasmid transfer-constitutive phenotype in strain K588, we constructed a cosmid library of pTiK588 by ligating the HindIII partially digested plasmid to the cosmid vector pLAFR3. The library was introduced to strain A6, and the transconjugants constitutively producing the QS signal were screened. One transconjugant, which contained the cosmid clone pBK129, was found to produce a similar level of AHL signals as strain K588 in the absence of octopine. The insert of pBK129 was sequenced, and analysis of the DNA sequence (GenBank accession no. DQ195264) showed that it contained about 26 kb of nucleotides, including 24 intact genes ranging from *traD* to *mclB* as well as a part of *traG* (27). These genes were positioned in the same order as their counterparts of pTiR10, a wild-type Ti plasmid of octopine strain R10 (40). Moreover, as expected, the whole insert region showed a high degree of homology (99%) at the nucleotide level with the same region in pTiR10. Nevertheless, nucleotide variations were identified in 11 of the 24 genes, including *traA*, *traF*, *traB*, *traM*, *ophEDCBA*, *msh*, and *ooxA*. Interestingly, among the several key genes known to be involved in regulation of both QS signal production and Ti plasmid conjugative transfer, including *occR*, *traR*, and *traM*, only the *traM* of pTiK588 contained three nucleotide variations (T161C, T268A, A269T), which resulted in two amino acid substitutions (L54P, Y90I), in comparison with the *traM* of pTiR10 (10). We also amplified and sequenced the corresponding genes from strain A6 and found that the *occR*, *traR*, and *trlR* genes are identical between strains K588 and A6. However, the *traM* gene of A6 also contained the T268A and A259T variations, indicating that only one single nucleotide mutation (T161C), which caused leucine-to-proline substitution at the 54th amino acid (L54P) of the 102-amino acid TraM, was worthy of further investigation. The data seem to be agreeable with the previous finding that substitution of Leu54 with alanine of the TraM of pTiR10 by site-directed mutagenesis could result in partial reduction in inhibitory activity against the TraR-mediated reporter gene expression (29). It is important that the cosmid pBK129 carries both *traM* and *traR*. Previous study showed that mutations in *traM* lead to constitutive expression of the TraR-AHL-dependent *tra* genes in the absence of conjugative opines (10, 14), indicating that the strains lacking a functional TraM could produce a critical amount of TraR and AHL for *tra* gene expression. The DNA sequence analysis suggested that pBK129 may encode a nonfunctional TraM but produce a functional TraR, which may well explain why introduction of the cosmid into strain A6 resulted in constitutive production of AHL signals.

Expression of the *traM* from strain A6 rescued the QS-constitutive phenotype of strain K588. To test whether the L54P mutation in homogenously expressed TraM was responsible for the QS-constitutive phenotype in strain K588, we cloned the wild-type *traM* gene from *A. tumefaciens* strain A6 ($traM_{A6}$) and tested whether it could effectively rescue the QS-constitutive phenotype of K588. The construct pLA-$traM_{A6}$-PT, in which the $traM_{A6}$ gene was controlled under its cognate promoter, was introduced into strain K588, and then AHL production was measured. As shown in Fig. 2A, in the absence of octopine induction, the amount of AHL produced on the solid agar plate by K588(pLA-$traM_{A6}$-PT) was dramatically decreased to almost the same level as that of wild-type strain A6. Consistently, we were also unable to detect AHL in the supernatant of K588(pLA-$traM_{A6}$-PT) cell culture. Moreover, expression of $traM_{A6}$ abolished the constitutive Ti plasmid conjugative transfer of K588 (Fig. 2B). As expected, the inhibition of AHL production and Ti plasmid transfer provided by $TraM_{A6}$ was significantly alleviated by supplementation of octopine (Fig. 2A and B).

Replacement of $traM_{A6}$ with $traM_{K588}$ in strain A6 did not generate the expected QS-constitutive phenotype. To further examine the consequence of L54P mutation in TraM, we cloned the *traM* gene from K588 (designated $traM_{K588}$) and used it to replace the A6 counterpart by gene replacement. The allele-exchanged strain, verified by DNA sequencing analysis, was designated A6(pTiA6$TraM_{K588}$) and subjected to phenotype analyses. Most surprisingly, however, strain A6(pTiA6$traM_{K588}$) failed to accumulate detectable AHL in the culture supernatant in the absence of octopine induction (Fig. 3A). When analyzed on the solid agar plates, we found that strain A6(pTiA6$traM_{K588}$) produced a basal level of QS

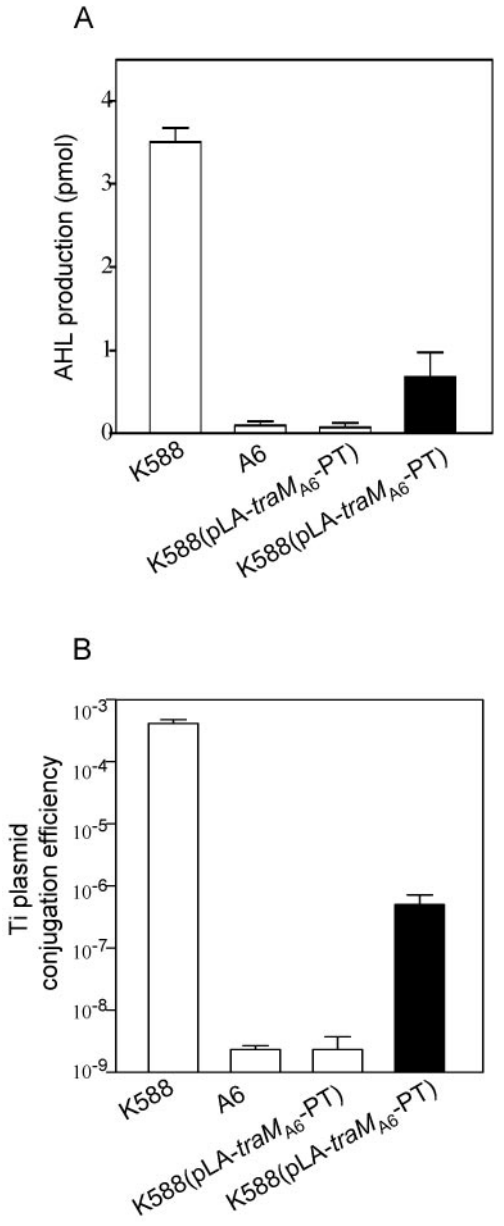

FIG. 2. The wild-type TraM from *A. tumefaciens* strain A6 rescued the QS-constitutive phenotype of strain K588. The assays were conducted in the absence (open bar) or presence (solid bar) of octopine. (A) AHL production by strain K588 and its derivatives on solid agar plate. (B) Ti plasmid conjugative transfer efficiency of the above strains. The donor cells were grown on a solid agar plate as described. Strain A6 was included as a control.

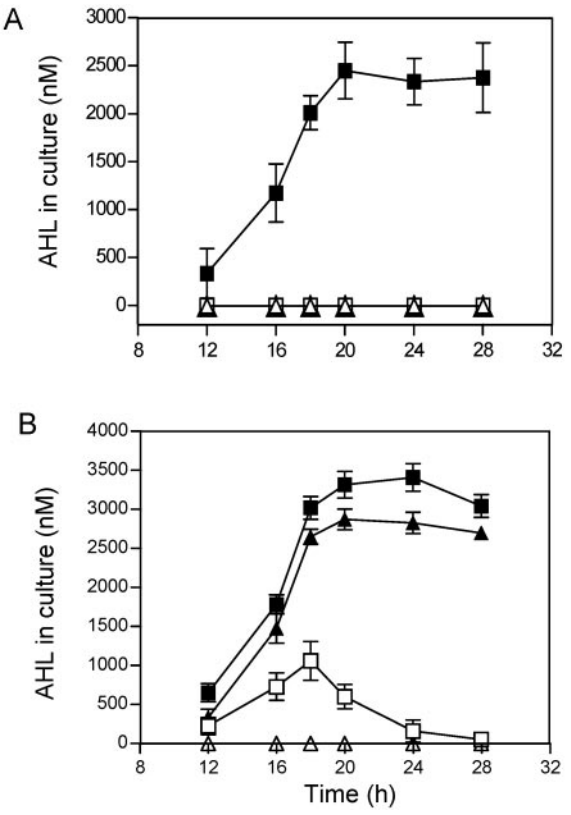

FIG. 3. Differential impact of TraM mutation on QS signal production by different *A. tumefaciens* strains in liquid culture. The assays were done with (solid symbol) or without (open symbol) octopine. (A) Effect of TraM point mutation (L54P) on AHL production by strain A6. Squares, A6(pTiA6*traM*$_{K588}$); triangles, A6. Note, data for A6 with and without octopine were identical, and as a result, solid triangles overlapped with open triangles. (B) Plasmid pTiA6*traM*$_{K588}$ displayed different AHL production phenotypes in *A. tumefaciens* strains C58C1RS and Ach5C3. Squares, C58C1RS(pTiA6*traM*$_{K588}$); triangles, Ach5C3(pTiA6*traM*$_{K588}$).

signal (0.15 ± 0.02 pmol) similar to that of wild-type strain A6 but much less than that of K588 (Fig. 1B).

When induced by octopine, however, the amount of AHL signals accumulated in the culture supernatant of strain A6(pTiA6*traM*$_{K588}$) reached about 2,500 nM at 20 h after inoculation (Fig. 3A), which was slightly lower than that of strain K588 (Fig. 1B). On solid agar plates supplemented with octopine, we found that strain A6(pTiA6*traM*$_{K588}$) produced a >40-times-higher amount of AHL signal than its parental strain A6 (75 ± 0.3 pmol versus 1.7 ± 0.1 pmol), which was at the same level as that of strain K588 (75 ± 0.6 pmol). It becomes highly intriguing that the L54P mutation in TraM, which results in the QS-constitutive phenotype in strain K588, is not able to change the QS-inducible phenotype of strain A6. The data strongly suggest the existence of critical genetic variations between strains K588 and A6 other than the difference in *traM*.

The differential impact of TraM mutation on QS in strains C58C1RS and A6 could be attributed to the chromosomal variations between the two strains. To probe whether the critical genetic variations are in pTiA6 or in the chromosomal background of strain A6, we transferred pTiA6*traM*$_{K588}$ from strain A6(pTiA6*traM*$_{K588}$) into the Ti plasmid-free nopaline-type strain C58C1RS by conjugative transfer, as described in Materials and Methods. The resultant transconjugant was designated C58C1RS(pTiA6*traM*$_{K588}$). The bioassay results showed that, unlike its donor strain A6(pTiA6*traM*$_{K588}$), C58C1RS(pTiA6*traM*$_{K588}$) displayed typical QS-constitutive phenotypes like K588, proficiently producing the AHL signals in liquid culture without octopine (Fig. 3B) and transferring Ti plasmid with high efficiency (data not shown), which were

comparable to strain K588 (Fig. 1B and D). Similarly, the AHL amount produced by C58C1RS(pTiA6*traM*$_{K588}$) could be further increased by octopine induction (Fig. 3B).

The above data strongly imply that an unknown factor(s) encoded by the A6 chromosomal background, which is missing in strains K588 and C58C1RS, is responsible for the differential impact of the L54P mutant in TraM on QS in different strains, such as A6 and K588. To test whether this phenomenon could also occur in other strains, we then transferred the plasmid pTiA6*traM*$_{K588}$ from A6 to Ach5C3, which is a Ti plasmid-free octopine-type *A. tumefaciens* strain. The transconjugants, designated Ach5C3(pTiA6*traM*$_{K588}$), were assayed for AHL production. The results in Fig. 3B show, interestingly, a pattern similar to that of pTiA6traM$_{K588}$ in the genetic background of strains A6, with no QS signal production in the absence of octopine (Fig. 3A). Based on these data, we concluded that octopine strains, at least A6 and Ach5C3, but not the nopaline strain C58, might contain a gene(s) that encodes an unidentified factor(s) suppressing QS signal production, directly or indirectly, in the absence of opine inducer.

The unknown factor in octopine strain A6 regulates *traI* expression at the transcriptional level. It remained to be determined whether the inhibitory effect of the unknown factor on QS signal production and conjugation in octopine strains was exerted at the transcriptional or translational level. For this purpose, we used RT-PCR to analyze the transcriptional expression patterns of *traI*, which is located on the Ti plasmid and encodes synthesis of the QS signal AHL in *A. tumefaciens* (15, 36), in different chromosomal genetic backgrounds and with or without octopine. As shown in Fig. 4A, the *traI* transcriptional expression in strain A6(pTiA6*traM*$_{K588}$) was low without octopine but was significantly promoted by the addition of the opine inducer. While in the case of C58C1RS(pTiA6*traM*$_{K588}$), the *traI* transcription maintained a high level regardless of whether there was octopine in the medium or not. The RT-PCR results were further confirmed by real-time RT-PCR analysis (Fig. 4B). By normalizing the abundance of *traI* transcripts of the bacterial cells grown with or without octopine to the amount of 16S rRNA in the same set of sample, we showed no significant difference in *traI* transcription by strain C58C1RS(pTiA6*traM*$_{K588}$) under both conditions. However, in strain A6(pTiA6*traM*$_{K588}$), we found that octopine induction enhanced *traI* transcription by about 60-fold, reaching a level similar to that of strain C58C1RS(pTiA6*traM*$_{K588}$). The data confirm that the unknown factor in strain A6 inhibits *traI* transcriptional expression, either directly or indirectly, but that repression can be released by the conjugative opine.

Identification of *traM2* in *A. tumefaciens* A6. Interestingly, several phenotypes of this unknown factor are reminiscent of those of TraM. First, similar to the *traM* mutant of strains C58 and R10 (10, 14), overexpression of wild-type *traM*$_{A6}$ in strain K588 restored the QS-inducible phenotype (Fig. 2). Second, the unknown factor suppressed the transcriptional expression of *traI* in the absence of opine inducer (Fig. 4), which is again similar to that of TraM (14). It was, hence, highly pertinent to determine whether strains A6 and Ach5C3 had a *traM* homologue. Total DNA samples were prepared from several related *A. tumefaciens* strains and digested with BamHI. After separation by gel electrophoresis, the DNA samples were hybridized at low stringency with *traM*$_{A6}$ probe. Strains C58 and K588 produced one hybridization band about 2 or 1.5 kb in size (Fig. 5A), which was the expected DNA band size of *traM* in nopaline and octopine strains, respectively. Strain A6 contained two hybridization bands: one was the *traM* band about 1.5 kb in size and the second band was about 5 kb. Coincidently, strain Ach5C3, which contained the unknown factor as well (Fig. 3B), also produced the 5-kb band (Fig. 5A). The weak hybridization signal of the 5-kb band suggests that strains A6 and Ach5C3 may contain a second copy of *traM* (designated *traM2*) in the chromosomal DNA, which is homologous, but not identical, to the *traM* carried by the Ti plasmid.

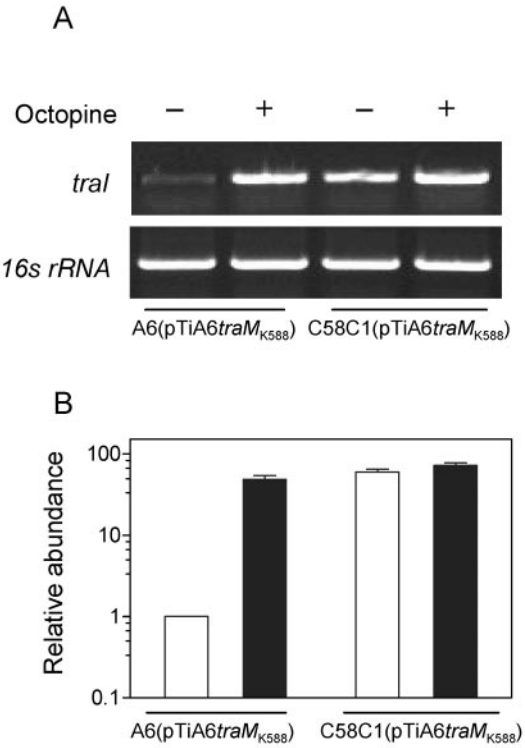

FIG. 4. Analysis of *traI* transcriptional expression in *A. tumefaciens*. (A) RT-PCR detection of *traI* transcripts in *A. tumefaciens* strains A6(pTiA6*traM*$_{K588}$) and C58C1RS(pTiA6*traM*$_{K588}$) with (+) and without (−) octopine induction. The 16S rRNA gene fragment was amplified under the same conditions as the internal control. (B) Real-time RT-PCR quantification analysis of the *traI* transcripts with (solid bar) or without (open bar) octopine. The bacterial strains were grown in BM liquid medium, and total cellular RNAs were extracted from bacterial cultures when the OD_{600} reached 1.2 and subjected to RT-PCR and real-time RT-PCR analysis as described in Materials and Methods. The relative amount of the *traI* transcripts was normalized to the 16S rRNA content within each reaction, and the analysis was repeated twice. For the convenience of comparison, the relative *traI* transcript abundance in strain A6(pTiA6*traM*$_{K588}$) without octopine induction was arbitrarily assigned a value of 1.

Assuming that *traM2* shares a reasonable degree of homology with the *traM* gene of Ti plasmid origin, several pairs of PCR primers were designed and tested in amplification of *traM2*, using the total DNA sample of strain A6 as a template. While the majority of primer pairs produced only one band with the expected size of *traM* amplification (data not shown), one pair of primers, which were designed based on the flanking

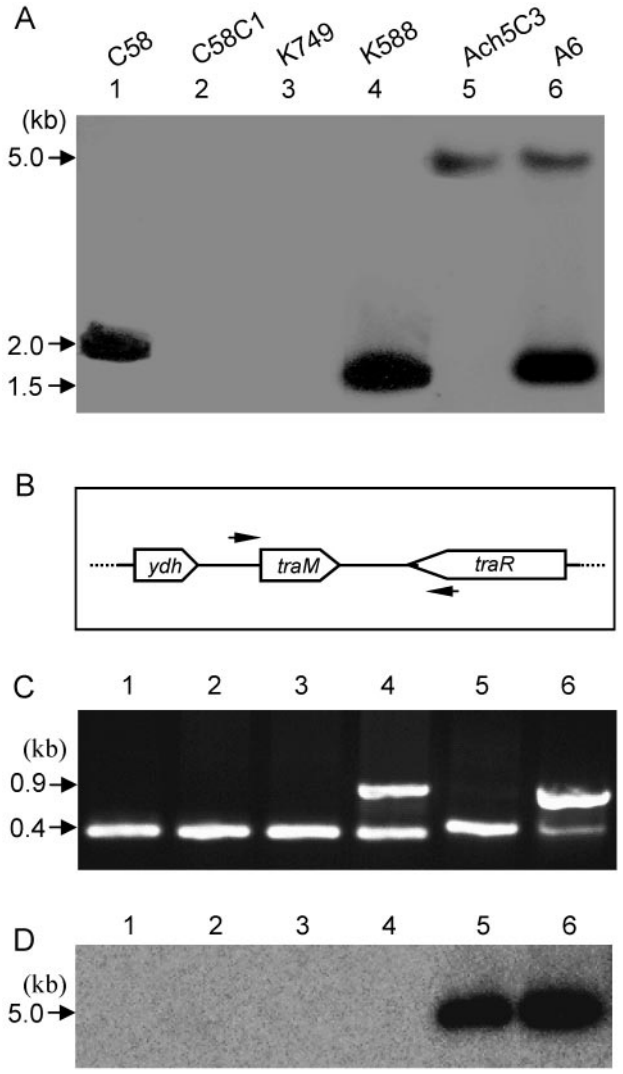

FIG. 5. Southern blot and PCR analysis of *traM* homologues in various *A. tumefaciens* strains. (A) Southern blot hybridization of nopaline-type strains (C58, C58C1RS, K749, and K588) and octopine-type strains (Ach5C3 and A6) using *traM* probe under low-stringency conditions (Materials and Methods). (B) Designing PCR primers to amplify the *traM* homologue from chromosomal DNA based on the *traM* flanking sequences in pTiA6. The relative locations of the final PCR primer pair used for amplification in panel C are indicated by arrows. (C) PCR fragments amplified from different strains as indicated in panel A. The 0.9-kb band was the expected DNA fragment amplified from individual octopine-type Ti plasmid, which was absent from three Ti plasmid-free strains, i.e., C58C1RS, K749, and Ach5C3. The band could not be amplified from Ti plasmid-containing nopaline strain C58 because of low homology in the region. The about 0.4-kb bands from strain A6 and Ach5C3 were shown to contain *traM* homologues. The similarly sized nonspecific bands were also amplified from the 4 nopaline-type derivatives (see text). (D) High-stringency Southern blot analysis using *traM2* as probe. In panels C and D, the samples used were the same as in panel A.

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   TraM MELEDANVTKKVELRPLIGLTRGLPPTDLE 30
TraM_K588 MELEDANVTKKVELRPLIGLTRGLPPTDLE
  TraM2 MDLKDSVVSDTFELRPVIGLTRGLSSADIE
        *:*:*: *:...****:*******..:*:*

   TraM TITIDAIRTHRRLVEKADELFQALPETYKT 60
TraM_K588 TITIDAIRTHRRLVEKADELFQAPPETYKT
  TraM2 TLTANAIRLHRQLLEKADQLFQVLPDDIKI
        *:* :*** **:*:****:***. *:  *

   TraM GQACGGPQHIRYIEASIEMHAQMSALNTLY 90
TraM_K588 GQACGGPQHIRYIEASIEMHAQMSALNTLY
  TraM2 GTAAGGEQHLEYIEAMIEMHAQMSAVNTLV
        * *.** **:.**** *********:***

   TraM SILGFIPKVVVN 102
TraM_K588 SILGFIPKVVVN
  TraM2 GLLGFIPKVSVN
        .:******* **
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FIG. 6. Sequence alignment of three TraM homologues used in this study. Alignment of amino acid sequences was performed using the computational program Clustalw (http://sbcr.bii.a-star.edu.sg/clustalw/). The key amino acid residues showing strong influence on inhibition activity against TraR-mediated gene activation, identified in a previous study (29), were indicated by a triangle above the sequence. $TraM_{K588}$ differs from TraM in only one amino acid (L54P).

sequence of *traM* (Fig. 5B), produced two PCR fragments of about 900 and 400 bp, respectively (Fig. 5C). We then used the same primer pair to test the total DNA samples of other strains and found that the 400-bp band was amplified from all the strains tested, including octopine-type A6 and Ach5C3 and nopaline-type C58, C58C1RS, K749, and K588 (Fig. 5C). Sequence analysis confirmed that the 400-bp PCR products from strain A6 and Ach5C3 contained an identical *traM2* gene (GenBank accession no. DQ192640), which showed 64% and 65% homologies with *traM* of strain A6 at the nucleotide and peptide levels, respectively. In contrast, the similarly sized PCR product from C58 and its derivatives C58C1RS, K749, and K588 corresponded to an intergenic region on the linear chromosome of C58. The region contains two truncated open reading frames (Atu3178, Atu3179) (http://www.ncbi.nlm.nih.gov/), but none of them showed homology to *traM* (data not shown). High-stringency Southern blotting analysis using *traM2* as probe further confirmed that, among the six strains tested, only A6 and Ach5C3 contained the *traM2* gene (Fig. 5D). We further determined the presence of *traM2* in other octopine strains, including Ach5, R10, ATCC 15955, B6, and B6S3, under the same high-stringency Southern blotting conditions. However, no *traM2*-specific hybridization signal was detected in all the tested strains except Ach5 (data no shown). The existence of *traM2* in strain Ach5 is expected, as strain Ach5C3 is a Ti plasmid-free derivative of strain Ach5 (Table 1).

TraM2 is a potent antiactivator in vitro. Sequence alignment showed that basically all the key amino acid residues, which significantly contribute to antiactivation activity against TraR (29), were conserved identically between TraM and TraM2 (Fig. 6). The data suggest that TraM2 might have a potency similar to that of TraM in the formation of an antiactivation complex that prevents TraR from binding to its target promoter. As expected, our gel mobility assay (Fig. 7) showed that TraM2 of A6 was able to block the binding of TraR to the

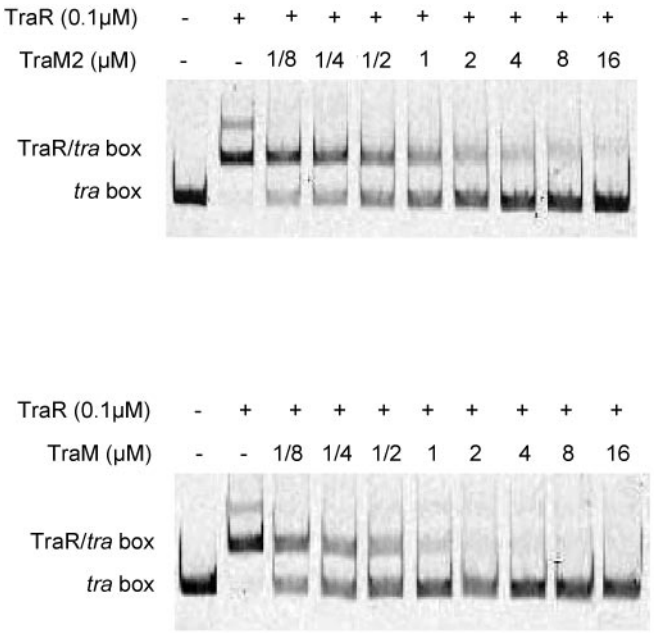

FIG. 7. Inhibitory effect of TraM2 on DNA binding to TraR by gel mobility assay. The inhibition potency of TraM2 from strain A6 (A) is compared with that of $TraM_{R10}$ (B). Concentrations of proteins (TraR, TraM, and TraM2) in the reaction mixtures are indicated above the nondenaturing gels (8%). Positions of the IR-labeled *tra* box in both free and complexed with TraR forms are indicated. At 16 μM, TraM2 displaced TraR completely from the *tra* box, compared to a lower concentration (~2 μM) of TraM. −, not included in the reaction mixture.

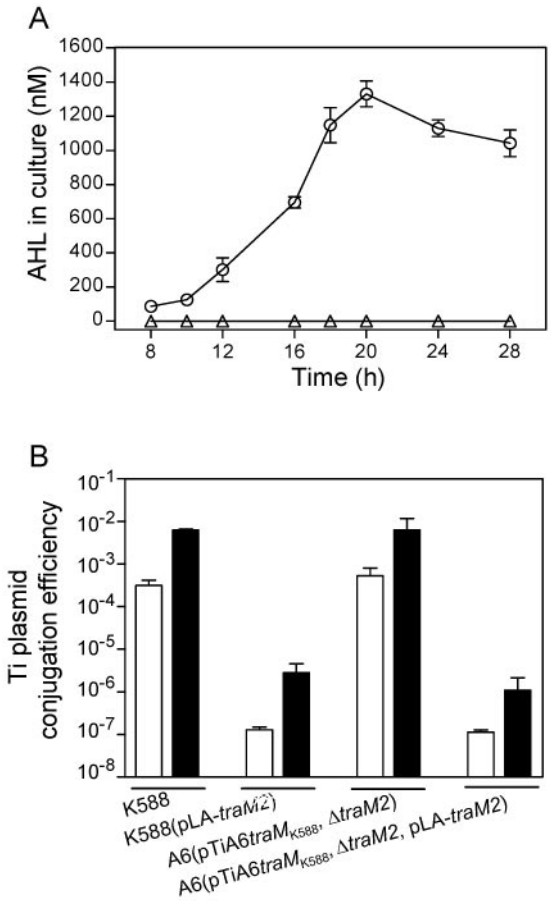

FIG. 8. Effect of null mutation of *traM2* on quorum sensing in *A. tumefaciens* strain A6. (A) AHL accumulation by *A. tumefaciens* strains A6(pTiA6$traM_{K588}$) (triangle) and A6(Δ*traM2*, pTiA6$traM_{K588}$) (circle) in the supernatant of bacterial culture without octopine induction. (B) Ti plasmid conjugative transfer efficiency of *A. tumefaciens* strains with (solid bar) or without (open bar) octopine induction on solid medium. Value are presented as means ± standard deviations of the results from at least three independent repeats.

tra box almost as effectively as the cognate antiactivator TraM. Thus, like TraM, TraM2 is a potent antiactivator to TraR under in vitro conditions.

TraM2 is a functional quorum-sensing modulator in vivo. To determine the physiological effect of *traM2* in strain A6, we generated a *traM2* deletion mutant in the genetic background of strain A6(pTiA6$traM_{k588}$), in which a central region of 234 nucleotides (corresponding to 78 amino acids) of *traM2* was deleted [designated A6(Δ*traM2*, pTiA6$traM_{k588}$)]. As described above, strain A6(pTiA6$traM_{k588}$) was not able to produce detectable AHL signals; however, deletion of *traM2* from the strain resulted in constitutive production of AHL in the absence of octopine inducer (Fig. 8A). Moreover, this *traM* and *traM2* double mutant A6(Δ*traM2*, pTiA6$traM_{k588}$) displayed a constitutive phenotype in Ti plasmid conjugal transfer, similar to that of strain K588 (Fig. 8B). We further showed that heterogeneous expression of *traM2* under the control of the constitutive *lac* promoter in both strains K588 and A6(Δ*traM2*, pTiA6$traM_{k588}$) abrogated the transfer-constitutive phenotype (Fig. 8B). The data demonstrate that TraM2 is involved in modulation of quorum sensing in strain A6.

DISCUSSION

The QS antiactivator TraM encoded by the Ti plasmid was discovered about a decade ago in *A. tumefaciens* nopaline-type strain C58 and octopine-type strain R10 consecutively by two laboratories (10, 14). These researchers found that transposon knockout of *traM* in both strains resulted in the constitutive production of AHL signals and high-efficiency transfer of Ti plasmid even in the absence of corresponding opine inducers. In this study, we showed that a single amino acid mutation in TraM, in which leucine 54 was replaced with proline, could fully account for the constitutive phenotypes of strain K588, a spontaneous mutant that produces AHL signals and transfers Ti plasmid efficiently without the need for octopine inducer (34). Our findings are highly consistent with the alanine scanning mutagenesis analysis of the TraM from pTiR10, which showed that an L54A substitution in TraM basically abolished its inhibitory activity against TraR-mediated gene expression when TraR was expressed at a low level, although the impact of L54A mutation on anti-TraR activity was reduced when TraR was expressed at a high level (29). The crystal structure of TraM revealed that the L54 residue is located at the end of an α-helix and lies on a region potentially interacting with TraR (4, 30). Replacement of L54 with proline may cause more severe conformational changes than with alanine, as proline is conformationally restricted in a peptide and consequently could have severe effects on the secondary and tertiary

structure of the protein. Our results have verified that under the native TraR expression status, the L54P substitution in TraM is sufficient to switch the Ti plasmid from QS-inducible mode to QS-constitutive mode in the chromosomal background of strain C58C1RS.

Surprisingly, however, we failed to generate the expected QS-constitutive phenotypes in octopine strain A6 after we replaced its wild-type *traM* with the mutated *traM* from pTiK588, which contained the L54P substitution (Fig. 3A). The same allele-exchanged Ti plasmid, when residing in the genetic background of nopaline strain C58C1RS, however, produced the QS-constitutive phenotype (Fig. 3B), similar to strain K588 (Fig. 1B). Subsequent genetic and biological analysis revealed that there is a second copy of *traM* carried by the chromosomal DNA of two octopine-type strains of *A. tumefaciens*, i.e., A6 and Ach5C3. The homologue, designated *traM2*, shares 64% and 65% identity with *traM* at the nucleotide and peptide levels, respectively. Although the identity between TraM and TraM2 is not particularly high, the key amino acid residues contributing to the antiactivation activity of TraM as revealed by alanine scanning analysis, including L29, A36, I37, H40, R41, L43, L54, A81, Q82, L93, G94, and P97 (29), are all conserved in TraM2 (Fig. 6). These data suggest that TraM2, similar to TraM, could also function as a potent QS antiactivator.

The previous results showed that TraM exerts its effect by direct interaction with the transcriptional factor TraR, which serves as the receptor of AHL QS signal and the key activator of the QS-dependent *tra* regulon (26, 42). The complex is highly stable, with a dissociation constant in the nanomolar range (29). DNA gel retardation analysis confirmed that TraM2, similar to the TraM encoded by the Ti plasmid, could effectively prevent TraR from binding to a *tra* promoter (Fig. 7), indicating that in vivo TraM2 might repress the QS-dependent expression of *tra* genes by interacting with the TraR activator encoded by pTiA6. The physiological effect of TraM2 has been demonstrated by deletion analysis. While the *traM* single mutant A6(pTiA6$traM_{k588}$) was dependent on octopine inducer for activation of AHL production in liquid culture (Fig. 3A), the double mutant A6(Δ*traM2*, pTiA6$traM_{k588}$) displayed a QS-constitutive phenotype in AHL production and Ti plasmid conjugal transfer (Fig. 8). The genetic data are consistent with the DNA gel retardation analysis that TraM2 is a strong antiactivator.

Even though mutation of the *traM* of pTiA6 along failed to change the QS-inducible phenotype (Fig. 3A), the role of its product in modulation of QS signaling in strain A6 is beyond any doubt. The wild-type strain A6 was unable to produce detectable AHL signals in liquid culture supplemented with octopine inducer; however, introduction of the L54P mutation in TraM enabled the strain to produce abundant AHL signals when induced by the conjugative opine (Fig. 3A). This is consistent with the demonstrated role of TraM in other bacterial strains (10, 14). All things considered, we can conclude that, together with TraM, TraM2 provides additional control to modulate the TraR-AHL-dependent activation of *tra* regulon in *A. tumefaciens* strain A6.

However, TraM2 does not seem to be a widely conserved quorum-sensing component in *A. tumefaciens*. From a limited *A. tumefaciens* strain collection, we found that the *traM2* gene exists only in octopine-type strains A6, Ach5, and its Ti plasmid-free derivative Ach5C3 but neither in the other tested octopine-type strains, i.e., R10, ATCC 15955, B6, and B6S3, nor in the nopaline-type strain C58. The absence of *traM2* in strain R10 is consistent with the previous finding that a null mutation of the *traM* carried by pTiR10 in octopine strain R10 resulted in constitutive Ti plasmid conjugative transfer (14), a phenotype typical to the *A. tumefaciens* strains that contain only one copy of *traM* (10).

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