JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1367–1373 Vol. 177, No. 5
0021-9193/95/$04.00+0

Activity of the *Agrobacterium* Ti Plasmid Conjugal Transfer Regulator TraR Is Inhibited by the Product of the *traM* Gene

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Received 17 October 1994/Accepted 19 December 1994

The *Agrobacterium* Ti plasmid *tra* regulon was previously found to be positively regulated by the TraR protein in the presence of a diffusible *N*-acyl homoserine lactone designated *Agrobacterium* autoinducer (AAI). TraR and AAI are similar to LuxR from *Vibrio fischeri* and the *Vibrio* autoinducer (VAI), which regulate target bioluminescence (*lux*) genes in a cell density-dependent manner. We now show that *tra* genes are also regulated by a second protein, designated TraM, which acts to antagonize TraR-dependent activation. The *traM* gene is closely linked to *traR*, and the two genes are transcribed convergently. The predicted TraM proteins of two different Ti plasmids are 77% identical but are not significantly similar to other protein sequences in the database, and thus TraM may represent a novel regulatory protein. Null mutations in *traM* cause strongly increased conjugation, *tra* gene transcription, and AAI production. A functional copy of *traM* introduced into *traM* mutants decreased conjugation, *tra* gene transcription, and AAI synthesis. TraM inhibits transcription of *traA*, *traI*, and *traM*. Although *traM* was first identified by its octopine-inducible promoter, we now show that induction by octopine requires *traR*, strongly suggesting that TraR is the direct *traM* activator.

In addition to transferring oncogenic DNA to the nuclei of host plant cells and causing crown gall neoplasia, Ti plasmids of *Agrobacterium tumefaciens* are capable of autonomous conjugal transfer between bacterial hosts (17). It has been known for some time that conjugation is strongly stimulated by tumor-released opines, indicating that this process normally occurs only in proximity to crown gall neoplasia (23, 31). The conjugal transfer (*tra*) regulons of both octopine-type and nopaline-type Ti plasmids are coregulated with opine catabolic operons (15, 32). In octopine-type Ti plasmids, the OccR (stands for octopine catabolism regulator) protein positively regulates *tra* genes in the presence of octopine (25, 47), while in nopaline-type Ti plasmids, AccR (stands for agrocinopine catabolism regulator) represses *tra* genes in the absence of agrocinopines (4).

In both types of Ti plasmids, *tra* gene induction is mediated by a two-step transcriptional cascade (19, 28, 39). In the first step, a specific opine directly stimulates the transcription of a *tra* gene regulator designated *traR* (4, 19, 39). The TraR protein then directly activates at least three *tra* promoters (20). TraR is homologous to the LuxR protein of *Vibrio fischeri* and to other quorum-sensing prokaryotic proteins (21). TraR activity requires *N*-3-(oxo-octanoyl)homoserine lactone (49), also called AAI (for *Agrobacterium* autoinducer), while the activities of homologous regulators require structurally similar *N*-acyl homoserine lactones (21). AAI synthesis requires the product of the Ti plasmid *traI* gene, which is homologous to the putative synthases of *N*-acyl homoserine lactones from a number of other bacteria (19, 28). AAI diffuses across the bacterial envelope in such a way that intracellular pools of this pheromone sufficient for *tra* expression are normally attained only if the bacteria are cultured at high cell density. In the laboratory, efficient conjugation of a wild-type octopine-type Ti plasmid requires either high donor cell densities or exogenous AAI (20). Under both conditions, octopine is also required to induce *traR* expression.

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Prior to the discovery of TraR or TraI, we used the promoter probe transposon Tn*5-gusA7* to isolate a collection of fusions between *gusA* and octopine-inducible *A. tumefaciens* promoters. One of these insertions was localized on the Ti plasmid and, quite unexpectedly, caused a strongly elevated frequency of Ti plasmid conjugal transfer (19). This insertion was tightly linked to the *traR* gene and led ultimately to the elucidation of *tra* gene regulation for octopine-type Ti plasmids. However, it remained unclear for some time why this insertion enhanced conjugation frequencies. In the present study, we provide evidence that the transposon insertion carried by this mutant disrupts a gene designated *traM*, which encodes a protein that negatively regulates conjugation and *tra* gene expression. This negative regulator is also found in nopaline-type Ti plasmids (27), but similar proteins have not yet been described for other quorum-sensing regulatory systems.

MATERIALS AND METHODS

Strains, plasmids, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. For *Escherichia coli* strains, standard antibiotic concentrations and growth conditions were used. *A. tumefaciens* strains were grown at 30°C in either Luria-Bertani broth or AT minimal broth (45) with appropriate antibiotics. Standard nitrogen and glucose concentrations in minimal media were 15 mM $(NH_4)_2SO_4$ and 0.5% glucose (ATGN). Octopine was obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and added to ATGN minimal medium at 0.8 mg of octopine per ml (ATGNO) for *tra* gene induction and at 2 mg/ml when used as a carbon and nitrogen source in minimal medium (ATO). Antibiotic concentrations used for *A. tumefaciens* were as follows: 150 μg of kanamycin per ml, 100 μg of gentamicin per ml, 50 μg of spectinomycin per ml, 500 μg of streptomycin per ml, 4.5 μg of tetracycline per ml, and 50 μg of rifampin per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Glu) were obtained from Gold Biotechnology (St. Louis, Mo.). *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) and *p*-nitrophenyl-β-D-glucuronide (NPG) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs (Beverly, Mass.).

Plasmids pCF218, pCF251, and pCF327, the first two of which have been described previously (19), were used to provide the *traR* gene in multicopy. Plasmid pCF327 contains a fusion between the *occQ* promoter and the *traR* structural gene (separated by 208 bp) (20). This plasmid also contains the constitutive *occRE23G* gene (7), which results in constitutive transcription of the *occQ* promoter and thus provides constant low-level expression of *traR*. Of the three plasmids, pCF218 produces the highest levels of the TraR protein, while pCF251 produces intermediate levels and pCF327 produces the lowest levels.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant features	Source or reference
Strains		
E. coli		
JM101	α-Complementation	35
SM10/λ*pir*	*pir* (supports R6K replication); *tra* genes from RK2	36
A. tumefaciens		
R10	Octopine-type strain; pTiR10; Occ^+	S. K. Farrand
A136	Ti plasmid-less derivative of C58; Rif^r Nal^r	42
C58 C1RS	Ti plasmid-less conjugal recipient; Sm^r Rif^r Occ^-	15
KYC6	R10 *traM6*::Tn*5-gusA7* Km^r	19
R1	R10 Φ(*traR-lacZ101*) Km^r	19
FPA26	R10 Φ(*traI26*::Tn*5-gusA7*) Km^r	19
WCF28	R10 *traM*::pCF346 Sm^r	This work
WCF29	KYC6 *traR*::pCF347 Km^r Sm^r	This work
Plasmids		
pTZ18R and pTZ19R	ColE1 plasmid vectors	U.S. Biochemicals
pCGN1558 and pCGN1559	*Agrobacterium* vectors containing Ri plasmid origin	33
pKNG101	*sacB* suicide plasmid for Campbell insertions	29
pSW213	IncP plasmid vector	6
pDH99	IncW plasmid vector	26
pJA411.62	1.5-kb *Bam*HI-*Eco*RI fragment from pTi15955 containing *traM*	J. Alt-Mörbe
pCF211	*traM6*::Tn*5-gus* cloned as an *Eco*RI fragment in pSW213	19
pCF212	*traM6*::Tn*5-gus* cloned as a *Kpn*I fragment in pSW213	19
pCF218	*traR* expressed constitutively from P*tetR*; cloned in pSW213	19
pCF251	*traR* expressed constitutively from P*tetA*; cloned in pDH99	19
pCF327	*traR* expressed constitutively from P*occQ*; cloned in pDH99	This work
pCF240.113	*traA113-lacZ* fusion cloned in pSW213	19
pCF346	pKNG101-derived suicide plasmid for *traM* disruption	This work
pCF347	pKNG101-derived suicide plasmid for *traR* disruption	This work
pMB100	*traM* cloned in pSW213	This work
pMB105	*traM* cloned in pCGN1558	This work

These conclusions are based both on Western immunoblotting experiments (37) and on the plasmids' ability to stimulate conjugal transfer efficiencies (20).

Genetic manipulations and mutagenesis. Plasmids were mobilized into *A. tumefaciens* strains via electroporation (34) or conjugal transfer from appropriate *E. coli* donor strains (36). Disruption of *traR* and *traM* on the Ti plasmid was performed by Campbell-type, homologous recombination-dependent plasmid insertion (36) by using internal fragments of each gene generated by PCR. The primers used for PCR were (i) 5′-GGCGCTAGCGAACTGGAAGATG CAAACGTGAC-3′ and 5′-GGCGGATCCCGTGCATCTCTATGCTCGCC-3′ to amplify a 238-bp *traM* internal fragment, and (ii) 5′-CCTCTAGATATGC CTACCTTCATATCCAG-3′ and 5′-CGCGGATCCGCGGACGCTGTTGTA CTTGAC-3′ to amplify a 468-bp *traR* internal fragment. PCRs were performed with a Labnet Hybaid thermal reactor for 30 cycles under standard reaction conditions (22). Amplification products were ligated into the pKNG101 suicide vector cleaved at its *Xba*I and *Bam*HI sites. The pKNG101 derivatives obtained, pCF346 and pCF347, were introduced by transformation into *E. coli* SM10/λ*pir* and transferred by conjugation into *A. tumefaciens* strains (36). Following overnight conjugation, dilutions of each mating were plated on ATGN agar containing streptomycin to counterselect against the auxotrophic *E. coli* SM10/λ*pir* donor and select for transfer of the suicide plasmid. Sm^r recombinants were isolated and subsequently scored for the presence of the pKNG101 *sacB* gene by screening for sensitivity to 5% sucrose (29). Sucrose-resistant derivatives of these mutants were selected and screened for streptomycin sensitivity and reversion to the wild-type phenotype via Campbell-type excision of the integrated plasmid.

DNA manipulations and sequencing. DNA manipulations were performed as described in standard protocols (41). Plasmid DNA was purified in single-stranded form for DNA sequencing by published procedures (35). DNA adjacent to the point of insertion of *traM6*::Tn*5-gusA7* was sequenced by using oligonucleotides complementary to the ends of the transposon, while DNA further from the transposon insertion was sequenced by using custom-made oligonucleotides (Cornell Biotechnology Center). Dideoxy chain termination reactions were performed with Sequenase version 2 (U.S. Biochemical Corp.) and α-^{35}S-ATP (Amersham Corp.). DNA sequence analysis was performed by using GenePro 4.2 (Riverside Scientific) and the University of Wisconsin Genetics Computer Group programs (10).

Bioassay for AAI activity. Strains were analyzed for production of AAI by adding cell-free supernatants of turbid cultures to 3-ml cultures of strain A136(pCF240.113)(pCF251) in ATGN broth. Since pCF240.113 contains a *traA-lacZ* fusion and pCF251, to express *traR*, cell-free supernatants containing AAI stimulate the production of β-galactosidase activity (19).

Ti plasmid conjugal transfer assays. Assays were performed as previously described (19). Donor strains were cultured overnight with shaking at 30°C in ATGN broth and concentrated 20-fold. The Ti plasmid-less strain C58 C1RS was prepared in a similar manner from ATGN cultures grown in the presence of rifampin and streptomycin. Equal volumes of the donor and recipient cultures were mixed, placed on 0.2-μm-pore-size filters, and incubated on ATO agar for 15 h at 30°C. The cells were then resuspended in water, serially diluted, and plated on AT agar containing rifampin, streptomycin, and octopine (800 μg/ml). Conjugation efficiencies were calculated as the ratio of Occ^+ transconjugants per recovered donor.

Nucleotide sequence accession number. The *traM* DNA sequence reported here has been deposited in the GenBank database under accession number U16786.

RESULTS

A hyperconjugal transposon mutant. In an effort to identify *A. tumefaciens* genes whose expression was induced by octopine, strain R10 was mutagenized with the promoter probe transposon Tn*5-gusA7* (43), and 5,000 Km^r transconjugants were screened for octopine-inducible β-glucuronidase (GUS) activity. From this screen, we ultimately isolated 27 strongly octopine-inducible fusions (8). Since it was known that Ti plasmid conjugation is stimulated by octopine, we anticipated that a subset of these insertions might lie in *tra* genes and would therefore be phenotypically Tra^-. We screened each transconjugant for conjugal transfer of Occ (the ability to degrade octopine) and of Km^r (resistance to kanamycin, which is conferred by the transposon) and found them all to be proficient in conjugal transfer of at least one of these phenotypes (six Tn*5-gusA7* insertions were in *occ* and transferred Km^r). The

TABLE 2. Conjugal transfer efficiencies in *A. tumefaciens* strains

Strain	Genotype	Conjugal transfer efficiency[a]	
		−Octopine	+Octopine[b]
R10(pSW213)	Wild type	$<1.3 \times 10^{-8}$	4.4×10^{-3}
KYC6(pSW213)	*traM* null mutant	1.1×10^{-4}	1.8×10^{-2}
KYC6(pCGN1559)	*traM* null mutant	2.1×10^{-4}	1.4×10^{-2}
KYC6(pMB100)	*traM* null mutant; *traM* multicopy	5.8×10^{-8}	4.4×10^{-3}
KYC6(pMB105)	*traM* null mutant; *traM* multicopy	2.1×10^{-5}	1.6×10^{-3}
R10(pMB100)	*traM* multicopy	$<2.0 \times 10^{-8}$	$<2.0 \times 10^{-8}$

[a] Conjugal efficiencies were calculated as described in Materials and Methods (transconjugants per output donor).
[b] Two milligrams of octopine per ml of induction agar.

absence of conjugation-deficient mutants was somewhat surprising and suggests that our screening conditions were appropriate for only a subset of octopine-inducible genes. Even more surprising was the finding that one strain showed sharply elevated levels of conjugation (Table 2). Unlike the parent pTiR10 plasmid, conjugal transfer of Occ by this mutant was easily detected even in the absence of octopine. This strain, KYC6, transferred Occ and Km^r at the same frequency (data not shown), strongly suggesting that the transposon was localized on the Ti plasmid.

The KYC6 Tn*5-gusA7* insertion and flanking sequence was subcloned into the plasmid pTZ18R by digesting KYC6 and vector DNA separately with *Eco*RI or with *Kpn*I, ligating, and selecting for the Km^r of the transposon in *E. coli* transformants. The insertion was localized to a 15-kb *Eco*RI fragment and a 22-kb *Kpn*I fragment (19). Since overlapping restriction fragments of these sizes are found on octopine-type Ti plasmids (11), this provided further evidence that the insertion was localized on the Ti plasmid. We ultimately found that the insertion lies just to the left of the *traR* gene (Fig. 1) (19).

After further subcloning, 1.5 kb of DNA surrounding this

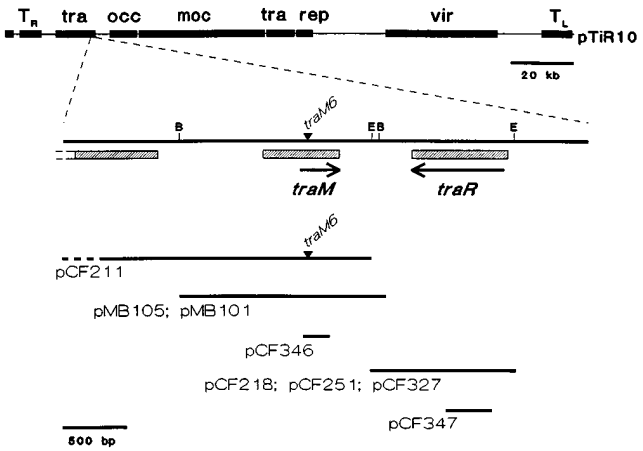

FIG. 1. Genetic map of the region of Ti plasmid pTiR10 containing *traM*. The top line represents the entire pTiR10 plasmid (see reference 23 for details). The second line is an expanded view of the region containing *traM* and *traR*. *traM6* is the point of insertion of transposon Tn*5-gusA7*. Hatched boxes represent DNA sequences that form heteroduplexes with the nopaline-type Ti plasmid pTiC58 (16). pCF211 is a cloned *Eco*RI fragment containing the *traM6* mutation. pMB100 and pMB105 contain the wild-type *traM* gene, while pCF218, pCF251, and pCF327 contain the wild-type *traR* gene. pCF346 and pCF347 contain PCR-amplified fragments used to disrupt wild-type *traM* and *traR* genes, respectively.

insertion was sequenced. The Tn*5-gusA7* insertion was located at codon 20 of a 102-codon open reading frame (Fig. 2A). By agreement with S. K. Farrand and colleagues, who recently discovered a similar gene in the nopaline-type Ti plasmid pTiC58 (18), we designate this gene *traM*. *traM* and *traR* are transcribed convergently (Fig. 1), with 472 bp separating their respective stop codons. The predicted *traM* translation product is composed predominantly of hydrophilic amino acid residues except for a short hydrophobic segment at its COOH-terminal end (Fig. 2B). Searches of the nucleic acid and protein sequence databases using Blast (1) and TFasta (38) failed to identify homologous proteins. Additionally, the Motifs program of the Genetics Computer Group software package did not reveal any recognizable sequence motifs.

To verify that the phenotype of the KYC6 mutant was not peculiar to the *traM6-gusA* insertion we generated an additional *traM* null mutant. A 268-bp internal fragment of the *traM* coding sequence, lacking the ribosome binding site and extending 21 codons from the 3′ end of *traM*, was obtained by PCR (see Materials and Methods). The amplification product was ligated into the pKNG101 suicide plasmid to construct pCF346, and this plasmid was used to generate the *traM* null mutant WCF28 by Campbell insertion into the resident *traM* gene of strain R10. WCF28 showed the same octopine-independent conjugation and AAI production as the KYC6 transposon mutant (data not shown).

The *traM* genes of the octopine- and nopaline-type Ti plasmids are 78% identical at both the nucleotide and protein levels (Fig. 2). Detectable DNA sequence homology ends precisely at the stop codons of the two genes. However, strong conservation extends approximately 300 bp upstream of their start codons, possibly due to the presence of *cis*-acting regulatory sites required for regulated *traM* expression. For example, nucleotides 872 to 888 of the R10 *traM* gene contain two copies of the sequence 5′-AGGGTTCA-3′, and virtually identical sequences were identified upstream of the pTiC58 *traM* gene (27). Additionally, the upstream conserved region also contains two sequences (nucleotides 634 to 651 and 727 to 744) showing moderate similarity to several putative TraR binding sites, the so-called *tra* boxes, previously described (Fig. 2A) (17, 26). These putative *tra* boxes flank a possible σ^{70}-type promoter sequence (nucleotides 671 to 699). Interestingly, the putative *tra* boxes and promoter are located within the 300 bp of upstream, noncoding sequence conserved between pTiR10 and pTiC58 (Fig. 2A). In fact, the upstream *tra* box-like element defines the 5′ boundary of this conserved region. Strong sequence similarity between the nopaline-type and octopine-type Ti plasmid regions including the *traM* genes was first predicted by heteroduplex mapping studies long before either gene had been identified (Fig. 1) (16).

The wild-type *traM* gene inhibits conjugal transfer. The octopine-type *traM* gene was isolated as a *Bam*HI-*Eco*RI fragment from plasmid pJA411.62 (Fig. 1) and was cloned into the broad host range plasmids pSW213 and pCGN1558, creating plasmids pMB100 and pMB105, respectively. Both plasmids decreased the conjugal efficiency of strain KYC6 in the presence and absence of octopine (Table 2), indicating that the *traM* transposon mutation in KYC6 was recessive in these merodiploid strains. The inhibitory effect of the *traM* gene on conjugal transfer was independent of the orientation of the *Bam*HI-*Eco*RI fragment in pSW213 (data not shown), suggesting that *traM* was expressed from a promoter element present on the cloned fragment. The KYC6 mutant synthesizes high levels of AAI activity in the absence of octopine (19). As might be predicted, pMB105 also caused a sharp decrease of AAI activity in KYC6 supernatants (data not shown). In the wild-

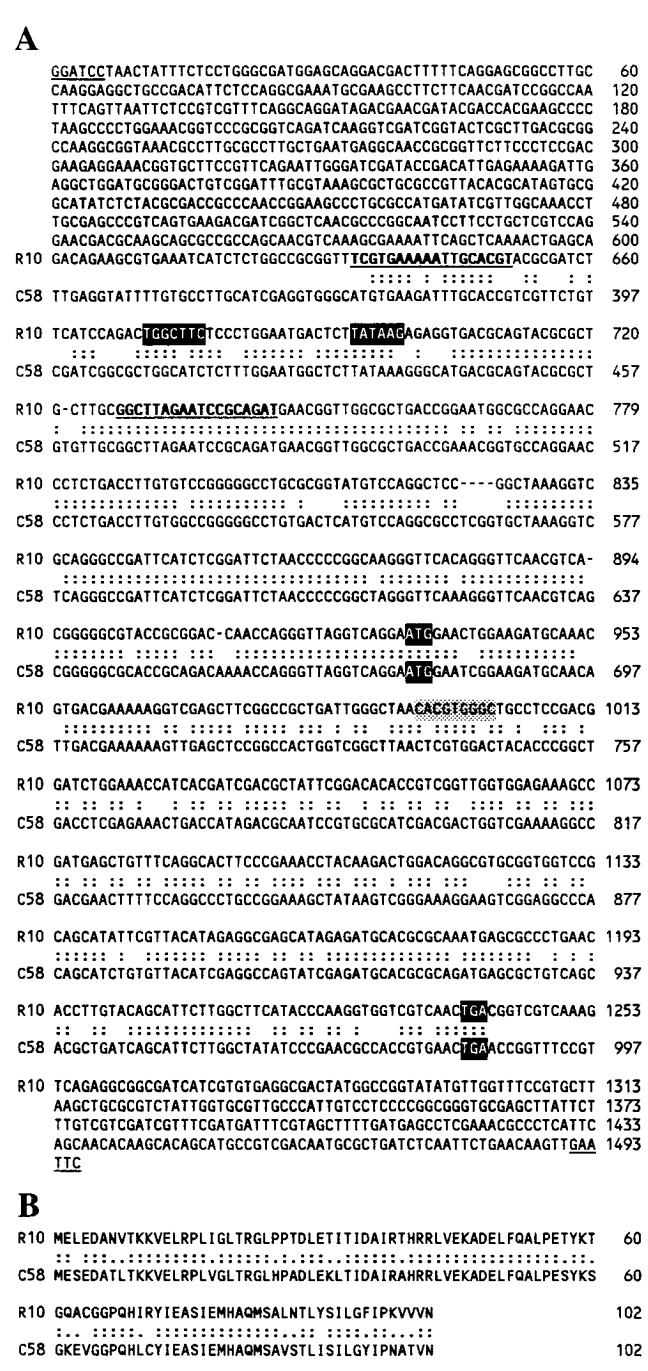

FIG. 2. DNA sequence of *traM* and homology with the *traM* gene of pTiC58. (A) DNA sequence of a fragment that encodes a functional *traM* gene, flanked by *Bam*HI and *Eco*RI endonuclease restriction cleavage sites (underlined). The 9-bp duplication caused by the Tn*5-gusA7* insertion is stippled. The −10 and −35 sequence elements of a possible σ^{70}-type promoter on the pTiR10 sequence (nucleotides 671 to 699), as well as the predicted ATG initiator codon and ATG termination codons from both pTiR10 and pTiC58 sequences, are boxed. Possible *tra* box elements on the pTiR10 sequence are underlined and in bold type. Sequence homology with *traM* from pTiC58 (GenBank accession number, L34744) (25) is indicated. Double dots indicate identical bases. (B) Protein sequence homology between the pTiR10 and pTiC58 TraM proteins. Double dots indicate identical amino acids, while single dots indicate conservative amino acid substitutions.

type *A. tumefaciens* R10 background, pMB100 abolished detectable conjugation of the Ti plasmid in the presence of octopine (Table 2). This indicates that elevating the *traM* gene dosage increases the inhibitory effects of this gene.

TraM does not alter induction of *traR* by octopine. Ti plasmid conjugation is completely dependent on the *traR* gene, which is activated by OccR in response to octopine (19). Perhaps the simplest way that TraM could act would be to decrease the induction of *traR* in response to octopine. To test this, the *traM*-expressing plasmid pMB105 was introduced into *A. tumefaciens* R1, which carries an octopine-inducible *traR-lacZ* fusion in place of the wild-type *traR* gene on the Ti plasmid (19). The expression of *traM* from pMB105 had no effect on the basal (3 Miller units) or octopine-induced (15 Miller units) levels of the *traR-lacZ* fusion.

TraM inhibits expression of TraR-regulated promoters. TraR and AAI activate at least four *tra* promoters, including P*traA* and P*traI* (19, 20), and TraM could act by inhibiting the transcription of one or more of these. To test this we measured *tra* gene expression in strains expressing elevated levels of TraM. Strain FPA26 carries a Tn*5-gusA7* insertion in the *traI* gene, does not synthesize AAI, and does not activate *tra* genes in the presence of octopine (19). However, *tra* expression in FPA26 can be activated by TraR in the presence of exogenous AAI. In order to measure the effect of TraM on expression of *traI* and *traA* simultaneously in a single strain, we introduced into FPA26 the plasmid pCF240.113, which carries a *traA-lacZ* transcriptional fusion (19), and extra copies of *traM* expressed from plasmid pMB105 (or a vector control). We used filtered, cell-free culture fluids from *A. tumefaciens* R10(pCF218), a preparation with high levels of AAI activity (19), as a source of crude AAI. This crude preparation has an AAI activity roughly equivalent to a 42 μM solution of pure, organically synthesized AAI as estimated by comparative dose-response curves with the AAI bioassay strain A136(pCF251)(pCF240.113). Although crude AAI was added in amounts that effectively induce expression of the *traA* and *traI* fusions in the FPA26 mutant, increased expression of *traM* in these strains strongly inhibited the activation of both promoters (Table 3).

In the experiment described above, *traR* was expressed at relatively low levels from its native promoter(s) on the Ti plasmid. To determine whether the inhibition of *tra* gene expression by TraM was affected by providing additional copies of *traR*, we added the multicopy plasmid pCF251, which expresses *traR* constitutively from the *tetA* promoter of pDH99 (19). pCF251 completely reversed TraM-mediated inhibition of P*traA* (Table 3). Therefore, inhibition of *tra* gene expression by TraM in FPA26 can be overcome by elevated levels of *traR*.

In order to determine whether TraM inhibition requires other Ti plasmid-encoded genes, similar assays were performed with derivatives of strain A136, which lacks a Ti plasmid. In these experiments plasmid pCF327 provided moderate constitutive levels of TraR (see Materials and Methods), and pCF240.113 allowed measurement of *traA-lacZ* expression. Diluted crude AAI was added exogenously, and pMB105 (or a vector control) was added to provide TraM. pMB105 caused a clear change in the response to low or moderate levels of AAI. More AAI (10,000-fold) was required for half the maximal activation of the *traA-lacZ* fusion in the presence of TraM than in its absence (Fig. 3). However, in the presence of very high levels of AAI, *traA-lacZ* expression was fully induced, indicating that TraM is not inhibitory under these conditions (Fig. 3). We conclude, first, that the inhibition of *tra* expression by TraM did not require any Ti plasmid-encoded genes except *traR*, and second, that inhibition could be overcome by using high levels of AAI.

TABLE 3. Inhibition of TraR-dependent transcription by *traM* in *A. tumefaciens* strains

Strain	Genotype	β-Glucuronidase sp act[a]		β-Galactosidase sp act[a]	
		−AAI	+AAI[b]	−AAI	+AAI[b]
FPA26(pCF240.113)(pCGN1558)	*traI26*::Tn*5-gusA7 traA113-lacZ*	3	61	202	4,000
FPA26(pCF240.113)(pMB105)	*traI26*::*Tn5-gusA7 traA113-lacZ*; *traM* multicopy	2	2	227	304
FPA26(pCF240.113)(pMB105)(pCF251)	*traI26*::*Tn5-gusA7 traA113-lacZ*; *traM* multicopy; *traR* multicopy	ND[c]	ND	224	4,307

[a] Miller units. All assays were performed in the presence of 800 μg of octopine per ml.
[b] Three percent crude AAI preparation.
[c] ND, not determined.

In earlier experiments, TraM failed to inhibit *tra* genes in strains that expressed increased levels of TraR (Table 3). To test whether this was the case in the Ti plasmid-less A136 strain, similar experiments were performed in which pCF327 was replaced with pCF251, which contains a P*tetA-traR* fusion. TraM did not inhibit *traA* expression in this genetic background (data not shown). These results are similar to those obtained with pCF251 in the *traI* null mutant FPA26 and again suggest that the inhibitory effect of *traM* can be titrated by increasing the level of *traR* expression.

Regulation of *traM* expression. The original Tn*5-gusA7* insertion in KYC6 was isolated because of its octopine-inducible β-glucuronidase activity (see Table 4). It seemed possible that this induction could be due to direct activation via OccR and octopine or that TraR and AAI might activate *traM* expression directly. If the latter is true, then a plasmid that expresses TraR at high levels would be predicted to increase *traM-gusA* expression, since similar properties have been observed with other *tra* promoters (19). Plasmid pCF218, which directs the synthesis of high levels of the TraR protein, strongly elevated expression of the *traM-gus* fusion in the presence or absence of octopine (Table 4). This suggests that TraR directly activates *traM* and that the induction of this gene by octopine requires TraR and AAI, whose levels are increased by octopine. Interestingly, the plasmid pCF251, which provides TraR at lower levels than pCF218 (19, 37), has only a minimal effect on *traM* expression (Table 4).

A requirement of *traR* for octopine-inducible *traM* expression was confirmed by testing octopine induction of *traM* in a *traR* null mutant. Strain WCF29 is a derivative of KYC6 in which *traR* is disrupted by Campbell insertion mutagenesis with the suicide plasmid pCF347. This *traR* mutation completely blocked *traM* induction by octopine (Table 4). Since TraM inhibits expression of other TraR-regulated promoters, it seemed plausible that *traM* might be autogenously regulated. To test this, *traM-gus* induction by octopine in a derivative of KYC6 containing pMB100 was measured. Supplying functional TraM protein reduced β-glucuronidase expression by approximately threefold (Table 4), indicating that the *traM* gene has an inhibitory effect on its own expression, similar to its effect on the induction of other *tra* genes.

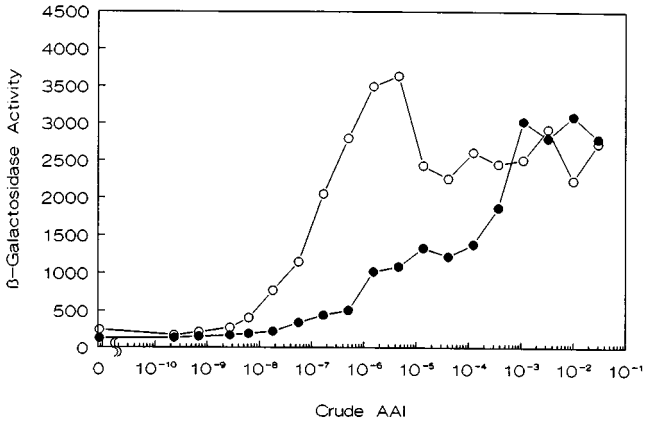

FIG. 3. Inhibition of *traA* expression by TraM. Derivatives of the Ti plasmid-less strain A136(pCF240.113)(pCF327) containing pMB105 (●) or pCGN1558 (○) were incubated for 15 h in different dilutions of crude AAI and assayed for β-galactosidase activity. pMB105 is a derivative of pCGN1558 expressing the TraM protein. Values are averages of the results of assays performed in triplicate.

DISCUSSION

The *tra* regulon of octopine-type Ti plasmids is controlled in a two-step transcriptional cascade requiring OccR, a member of the LysR family of prokaryotic regulators, and TraR-TraI, members of the LuxR-LuxI family of proteins. We now describe a negative regulator that does not share significant similarity with any known protein. Although a number of other quorum-sensing regulatory systems containing LuxR and LuxI homologs have been described (21, 44), none is known to have a protein that acts to antagonize the regulation of target genes by a LuxR homolog. However, there are examples of LuxR-type regulatory systems in which the expression of target genes is repressed in alternative ways. For instance, the *luxICDABEG* operon of *V. fischeri* is thought to be repressed two- to fivefold by the cyclic AMP receptor protein (CRP) in the absence of glucose (12–14). The mechanism of this mild CRP-dependent repression of the *lux* operon is unclear but requires the binding of cyclic AMP-CRP during activation of the divergently expressed *luxR* gene. Similarly, expression of the *rhi* genes of *Rhizobium leguminosarum* bv. leguminosarum is repressed by the NodD protein, but this functions by indirectly repressing

TABLE 4. Induction of *traM* by octopine in *A. tumefaciens* strains overexpressing *traM* or *traR*

Strain	Genotype	β-Glucuronidase sp act[a]	
		−Octopine	+Octopine[b]
KYC6(pSW213)	*traM6*::Tn*5-gusA*	34	290
KYC6(pCF218)	*traM6*::Tn*5-gusA*; *traR* multicopy	1,375	1,985
KYC6(pCF251)	*traM6*::Tn*5-gusA*; *traR* multicopy	73	338
WCF29	*traM6*::Tn*5-gusA* *traR*::pCF347	23	19
KYC6(pMB100)	*traM6*::Tn*5-gusA*; *traM* multicopy	31	97

[a] Miller units.
[b] Eight hundred micrograms of octopine per ml.

the expression of the LuxR-type transcriptional activator encoded by *rhiR* (9). Both CRP and NodD are global regulators whose regulation overlaps with the LuxR-type control, perhaps allowing fine tuning of the autoinduction response. In contrast, *traM* appears to be a specific antagonist for TraR-dependent activation and plays a key role in the AAI-dependent activation of Ti plasmid *tra* genes. It will be extremely interesting to determine whether other LuxR-type proteins also function in conjunction with regulatory antagonists.

The inhibitory role of *traM* is in some ways similar to the function of the *Vibrio harveyi luxO* gene, a specific repressor of *lux* gene expression (3). Although the *V. harveyi lux* genes are cell density regulated at least in part via the activity of an acylated homoserine lactone, the regulatory cascade appears to be remarkably different from the *V. fischeri* LuxR-LuxI system and its homologs. The proteins required for the synthesis and perception of *V. harveyi* cell density signals show no homology to the LuxR-LuxI family of proteins (2). In particular, the *luxO* gene product is a member of the family of two-component response regulators, is predicted to be a DNA-binding protein, and shares no homology with *traM*. While mechanistically the TraM and LuxO proteins appear to be unrelated, they may have similar functions.

There are many questions about TraM that remain unresolved. One important question concerns the mechanism by which it acts. Genetic evidence suggests that in the presence of AAI, TraR binds to conserved dyad symmetrical sequences called *tra* boxes found upstream of most or possibly all TraR-regulated promoters (19, 21, 24, 28). The simplest model for TraM function is that it in some way interferes with this process, either by binding to TraR (or AAI) and making it unavailable for activation or by binding to *tra* promoters in such a way as to prevent TraR functioning. Alternatively, TraM might degrade or modify TraR or AAI. We observe that the TraM-mediated antagonism can be overcome by high levels of either AAI or TraR (Fig. 3). Therefore, we prefer a model where TraM prevents either the formation of a TraR-AAI complex or, once formed, the activity of this complex. It may be possible to address this question by isolating alleles of *traR* or of *tra* promoters that are uninhibited by TraM. Reconstituting antagonism in vitro would provide the most definitive data about its mode of action, but this must await the reconstitution of TraR-mediated regulation.

One might also question what benefit Ti plasmids gain from the TraM antagonist. A long-standing conceptual problem with autoinduction is that as little as one molecule of *vibrio* autoinducer (VAI) per *V. fischeri* cell is sufficient to trigger *lux* gene activation (30). The fact that high cell densities are required for bioluminescence in *V. fischeri* and for conjugation in *A. tumefaciens* (20, 40) indicates that cells must be able to respond preferentially to exogenous autoinducer that accumulates only at high cell densities. How do the bacteria at low cell density prevent induction by endogenous autoinducers? TraM could play some role in preventing *tra* operons from being activated by the intracellular AAI synthesized by individual cells at low population densities. By raising the threshold of AAI required for induction, TraM could help to ensure that *tra* gene activation is quorum dependent. Such a role is suggested by the phenotype of *traM* null mutants, which are derepressed for conjugal transfer, *tra* gene expression, and AAI synthesis and appear to be short-circuited for normal regulation. Additionally, *traM* induction by TraR indicates that the pool size of TraM increases during *tra* gene induction and therefore increased levels of TraM could help return *tra* promoters to an uninduced state as AAI concentrations fall. It is also possible that inhibition by TraM is modulated by some environmental or physiological signal. Such a signal would thereby modulate conjugal transfer efficiencies.

The four proteins that control *tra* gene transcription might be viewed as constituting a delicately balanced genetic switch. This putative switch is known to be responsive to octopine and to high cell density (19); additional stimuli may await discovery. The existence of such a switch in turn suggests that a decision is made, on the basis of these stimuli, whether or not to induce the *tra* regulon. Conjugation may be considered an energetically expensive undertaking, especially in nutrient-limiting conditions as may prevail in the rhizosphere at high cell density. *tra* gene induction is reminiscent of the induction of the *vir* regulon elsewhere on the Ti plasmid, which is induced by another transcriptional cascade in response to at least three environmental stimuli (48). The *traM* gene is highly conserved between the octopine- and nopaline-type Ti plasmids, while the flanking sequence is more divergent (Fig. 1) (16), suggesting a strong positive selection for retention of TraM function. The fact that TraR induces the synthesis of its own antagonist indicates that *tra* gene regulation contains a negative autoregulatory loop. This is especially intriguing since the octopine-type *tra* regulon also has two positive autoregulatory loops, namely, the activation of both the *traR* and *traI* genes by TraR and AAI (19). This complex pattern of positive and negative autoregulation demonstrates similarities with the SOS and heat shock regulons of *E. coli*, both of which contain antagonistic positive and negative autoregulatory loops (5, 46).

ACKNOWLEDGMENTS

We thank Steve Farrand, Ingyu Hwang, and Dave Cook for sharing unpublished data and insights. Thanks also to Julianne Alt-Mörbe for pJA411.2.

This work was supported by National Institutes of Health grant GM42893 to S.C.W. and postdoctoral fellowship GM15128 to C.F.

Clay Fuqua and Michelle Burbea made equal contributions during this project.

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