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A LuxR-LuxI Type Regulatory System Activates *Agrobacterium* Ti Plasmid Conjugal Transfer in the Presence of a Plant Tumor Metabolite

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Conjugal transfer of *Agrobacterium* octopine-type Ti plasmids is activated by octopine, a metabolite released from plant tumors. Octopine causes conjugal donors to secrete a pheromone, *Agrobacterium* autoinducer (AAI), and exogenous AAI further stimulates conjugation. The putative AAI synthase and an AAI-responsive transcriptional regulator were found to be encoded by the Ti plasmid *traI* and *traR* genes, respectively, and the expression of *traR* was induced by octopine. The octopine-type *traR* gene product is highly homologous to the TraR protein recently characterized from a nopaline-type Ti plasmid. TraR and TraI are homologous to the LuxR and LuxI regulatory proteins of *Vibrio fischeri*, and AAI is similar in structure to the diffusable *V. fischeri* autoinducer, the inducing ligand of LuxR. TraR activated target genes in the presence of AAI and also activated *traR* and *traI* themselves, creating two positive-feedback loops. TraR-AAI-mediated activation in wild-type *Agrobacterium* strains was dramatically enhanced by culturing on solid media, suggesting a possible role in cell density sensing.

The exchange of chemical signals between bacteria has recently become an important theme in prokaryotic molecular biology (21, 42). Signal exchange is required for such diverse processes as fruiting body formation by myxobacteria (42), antibiotic production by actinomycetes (41), genetic competence and sporulation in *Bacillus subtilis* (36), colonization of the light organs of fish and squid by bioluminescent *Vibrio fischeri* (20), and conjugal transfer of bacterial plasmids between strains of *Enterococcus faecalis* (11). We describe a regulatory system that activates Ti plasmid conjugation between agrobacteria in response to chemical signals exchanged between donor cells.

The plant pathogen *Agrobacterium tumefaciens* causes crown gall tumors on higher plants by transferring discrete DNA fragments (T-DNAs) from its tumor-inducing (Ti) plasmid to the nuclei of infected cells (48, 72). T-DNA-encoded proteins direct the overproduction of the phytohormones auxin and cytokinin, as well as compounds, called opines, that are utilized by agrobacteria as nutrient sources (15). Ti plasmids also mediate their own conjugal transfer to Ti-plasmidless cells (44), and conjugation requires the presence of specific opines (34). Conjugation of the nopaline-type Ti plasmid pTiC58 is induced by agrocinopines A and B (24), while conjugation of broad-host-range octopine-type Ti plasmids is induced by octopine (45).

Conjugal transfer (*tra*) genes of the nopaline-type Ti plasmid pTiC58 are regulated by a diffusible compound termed conjugation factor and by a protein called TraR (55, 74). Conjugation factor [*N*-3-(oxooctanoyl)homoserine lactone] is an analog of the *V. fischeri* autoinducer (VAI), a diffusible compound that stimulates the luminescence (*lux*) operon of that organism. Because of its structural and functional similarities to VAI, we shall refer to conjugation factor as the *Agrobacterium* autoinducer (AAI). The nopaline-type Ti plasmid conjugal transfer regulator, TraR, is homologous to the *V. fischeri* LuxR protein (55). LuxR is the receptor for VAI (1, 39) and activates luminescence gene expression in the presence of VAI (26). The LuxR regulatory system acts as a cell density sensing mechanism (52). The similarities between TraR and LuxR and their inducing ligands suggest that Ti plasmid conjugation may be regulated in an analogous fashion. However, a LuxI homolog (the putative AAI synthase) has not been identified, and the regulation of *traR* and the AAI synthase gene remains undefined. In particular, the observation that octopine-type Ti plasmid conjugation and octopine catabolism are coregulated is largely unexplained. In this study, we address these issues by examining the control of conjugal transfer for an octopine-type Ti plasmid.

By screening for octopine-regulated genes, we identified the *traR* gene of octopine-type Ti plasmid pTiR10. This TraR protein is a member of the recently defined family of LuxR-type transcriptional activators (31) and is 81% identical to the TraR protein of pTiC58 (55). The *traR* gene is positively regulated by OccR, a LysR-type octopine-responsive protein that also activates octopine catabolic genes (37, 68). We identified the *traI* gene, which is required for AAI production, and found it to be homologous to the *V. fischeri luxI* gene. In addition to activating other *tra* genes, TraR and AAI also activate *traR* and *traI* themselves, creating a transcriptional switch similar to that of the *V. fischeri lux* regulators (26). Finally, conserved dyad symmetrical DNA sequences were found directly upstream of two TraR-AAI-regulated genes; these elements are similar to the LuxR binding sites of *V. fischeri*.

MATERIALS AND METHODS

Strains, plasmids, reagents, and genetic methods. The bacterial strains and plasmids used in this study are listed in Table 1. For all *Escherichia coli* strains, standard antibiotics and growth conditions were used, except that *E. coli* POI 1734 (9) was grown at 30°C. *A. tumefaciens* strains were grown at 30°C in either Luria-Bertani medium or AT (66) minimal salts medium with (i) 15 mM $(NH_4)_2SO_4$ and 0.5% glucose (ATGN) or with those two ingredients plus octopine (ATGNO) or (ii) with

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
Strains		
E. coli		
JM101	α-Complementation strain	50
MC4100	*lac* deletion strain	8
POI 1734	Mu*dJ* host strain	9
SY327/λ*pir*	Host for R6K plasmid maintenance	51
SM10/λ*pir*	Tra$^+$ R6K strain	51
A. tumefaciens		
R10	Octopine-type strain; pTiR10	S. K. Farrand
C58 C1RS	Ti plasmidless; Rif^r Sm^r	25
A136	Ti plasmidless; multidrug resistant	58
KYC6	(pTiR10::Tn*5-gusA7*) Km^r	This work
R1	ϕ(*traR-lacZ101*::LKC481) Km^r	This work
SR1	(*occR102*::ΩSp/Sm) Sp^r/Sm^r	This work
RO1	ϕ(*traR-lacZ101*::LKC481) Km^r (*occR102*::Sp/Sm Ω) Sp^r/Sm^r	This work
FPA26	ϕ(*traI-gusA26*::Tn*5-gusA7*) Km^r	This work
R10PI1	R10(pCF302 *traI-lacZ302*::KOK6) Km^r Sm^r *traI*$^+$	This work
Plasmids		
pTZ18R/19R	Phagemid sequencing vector	U.S. Biochemical Corp.
pSW213	IncP cloning vector	10
pDH99	IncW cloning vector	38
pVK200 library	pTiA6 cosmid library	46
pKNG101	R6K *sacRB* derivative; Sm^r	43
pCF116	R6K *sacRB* derivative; Km^r	This work
pKOK6	*lacZ*-Km^r operon fusion cassette	47
pLKC480 to pLKC482	*lacZY*-Km^r protein fusion cassettes	67
pSB504	Mobilizable Tn*5-gusA7* donor	61
pCF211	pSW213 (KYC6 14.7-kb *Eco*RI fragment [Tn*5-gusA7*])	This work
pCF212	pSW213 (KYC6 23.7-kb *Kpn*I fragment [Tn*5-gusA7*])	This work
pCF214	pSW213 (KYC6 3-kb *Hin*dIII fragment [1.1-kb IS*50*$_R$])	This work
pCF218	pSW213 (KYC6 1-kb *Eco*RI fragment)	This work
pCF251	pDH99 (KYC6 1-kb *Eco*RI fragment)	This work
pCF234	pSW213 (*traR* COOH-terminal deletion)	This work
pCF242	pSW213 (*traR Tth*111I fill-in, frameshift)	This work
pCF240	pSW213 (KYC6 10.8-kb *Kpn*I fragment)	This work
pCF278	pSW213 (pVK212 7.35-kb *Eco*RI fragment)	This work

octopine (2 mg/ml) as the sole source of carbon and nitrogen (ATO). Antibiotic concentrations used for *A. tumefaciens* were as follows: kanamycin, 150 μg/ml; rifampin, 50 μg/ml; streptomycin, 500 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 4.5 μg/ml. Octopine was purchased from Aldrich Chemical Co. 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. Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs. Synthetic *N*-acyl homoserine lactones were generously provided by A. Eberhard (Ithaca College) and E. P. Greenberg (University of Iowa).

Transposon mutagenesis. Mu*dlac* transposon insertions in *tra* genes were generated by transforming *E. coli* POI 1734 with pCF240, a broad-host-range plasmid containing *Kpn*I fragment 7 (18). pCF240 derivatives containing Mu*dlac* were isolated in *E. coli* MC4100 as described previously (9). β-Galactosidase assays were performed as described previously (38). Plasmids were introduced into *A. tumefaciens* by electroporation (49) or by conjugation (51). Tn*5-gusA7* transposon insertions were made with *E. coli* MC1061(pSB504) (61). pSB504 contains Tn*5-gusA7* on a mobilizable plasmid with the ColE1 replication origin, which is nonfunctional in *A. tumefaciens*. pSB504 was introduced into strain R10 by triparental mating (19), and transposition was selected by growth on minimal medium containing 200 μg of kanamycin per ml.

Recombinational mutagenesis. Marker replacement mutagenesis of the *traR* and *occR* genes was performed by positively selecting for allelic exchange (33). The coding sequences of the *traR* and *occR* genes were interrupted by in vitro insertion of either a *lacZY*-Km^r protein fusion cassette (67) or an Sp/Sm Ω cassette (29). The interrupted genes were introduced into a mobilizable suicide vector carrying the *sacRB* locus. The suicide plasmids used were pKNG101 (43) for the *traR* replacement and pCF116 for *occR* mutagenesis. *E. coli* SM10/λ*pir* strains harboring the pKNG101 or pCF116 derivatives were mated with *A. tumefaciens* strains on 0.2-μm-pore-size filters on ATGN agar plates overnight at 30°C. The cells were resuspended in water, serially diluted, and plated on ATGN agar containing kanamycin (to select for the *lacZY*-Km^r cassette) or spectinomycin (to select for the Sp/Sm Ω cassette) to select for homologous Campbell insertion of the mutagenic plasmids. Transconjugants were plated on ATGN agar containing the appropriate antibiotic and 5% sucrose to select for Campbell excision. Suc^r colonies were screened for loss of the plasmid marker. Candidates that were Suc^r, resistant to the

TABLE 2. Conjugal transfer efficiencies

Strain(plasmid)	Genotype or description	Conjugal transfer efficiency[a] in the absence (−) or presence (+) of octopine[b]	
		−	+
R10	Wild type	$<1.3 \times 10^{-8}$	1.0×10^{-4}
KYC6	Hyperconjugal mutant	5.8×10^{-4}	3.5×10^{-1}
R10(pSW213)	Vector control	$<2.0 \times 10^{-8}$	8.3×10^{-5}
R10(pCF211)	See Fig. 1	$<1.0 \times 10^{-8}$	2.2×10^{-4}
R10(pCF212)	See Fig. 1	6.0×10^{-6}	3.3×10^{-2}
R10(pCF214)	See Fig. 1	5.0×10^{-1}	7.0×10^{-1}
R10(pCF218)	See Fig. 1	2.2	1.6
R10(pCF251)	See Fig. 1	0.21	0.11
R10(pCF234)	*traR* COOH-terminal deletion	$<9.5 \times 10^{-7}$	1.3×10^{-4}
R10(pCF242)	*traR* frameshift	$<5.2 \times 10^{-7}$	8.1×10^{-5}
R1	*traR-lacZ101*	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
R1(pSW213)	*traR-lacZ101*; vector	$<2.5 \times 10^{-8}$	$<9.5 \times 10^{-8}$
R1(pCF218)	*traR-lacZ101*; *traR* (multiple copies)	0.11	0.2
SR1	*occR102*	$<3.5 \times 10^{-8}$	$<8.0 \times 10^{-7}$
SR1(pSW213)	*occR102*; vector	$<4.4 \times 10^{-8}$	$<9.1 \times 10^{-7}$
SR1(pCF218)	*occR102 traR* (multiple copies)	0.23	0.20
FPA26	*traI26*	$<1.3 \times 10^{-8}$	$<1.3 \times 10^{-9}$
FPA26(pCF278)	*traI26 traI* (multiple copies)	$<3.6 \times 10^{-9}$	$<2.8 \times 10^{-8}$

[a] Calculated as described in Materials and Methods.
[b] Two milligrams of octopine per ml of induction agar.

cassette-encoded marker, and sensitive to the plasmid marker were further characterized by Southern hybridization.

The *cis* merodiploid strain R10PI1 (*traI-lacZ302*) was constructed by in vitro insertion of a *lacZ* operon fusion cassette (47) at codon 108 in *traI* to create suicide plasmid pCF302 and then by Campbell insertion of pCF302 into pTiR10 as described above. Recombinants were screened for octopine-inducible expression of the *lacZ* reporter gene and verified by Southern hybridization (data not shown).

DNA manipulation and sequencing. DNA manipulations were performed by standard protocols (57). Single-stranded sequencing templates were generated from pTZ18/19R derivatives for sequencing with helper phage M13KO7 (50). The DNA sequence was obtained with universal and reverse primers as well as custom synthetic oligonucleotides purchased from the Cornell Biotechnology Center. Dideoxy chain termination sequencing reactions were performed with Sequenase version 2 (U.S. Biochemical Corp.) and [α-^{35}S]ATP (Amersham Corp.) as recommended by U.S. Biochemical Corp. GenePro 4.2 (Riverside Scientific) and the University of Wisconsin Genetics Computer Group (UWGCG) programs (16) were used for computer-assisted DNA sequence analyses. DNA sequences have been deposited in the GenBank data base under accession numbers L08596 (*traR*) and L17024 (*traI*).

Ti plasmid conjugal transfer assays. Donor strains were cultured overnight with shaking at 30°C in ATGN broth and concentrated 20-fold. The Ti-plasmidless 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, spotted onto 0.2-μm-pore-size filters, and incubated for 15 h at 30°C on petri dishes (50 by 12 mm) containing 5 ml of ATO agar. Following incubation, the filters were removed and washed thoroughly with 1 ml sterile H_2O to remove the cells. Serial dilutions of these washes were plated on selective media: Luria-Bertani agar plus rifampin, streptomycin, and kanamycin for transconjugants carrying Km^r-marked Ti plasmids and AT agar plus rifampin, streptomycin, and octopine (800 μg/ml) for transconjugants carrying unmarked Ti plasmids. The titer of each mating suspension was determined for the number of cells carrying the donor marker (Km^r or Occ$^+$), and conjugation efficiencies were calculated as the ratio of transconjugants per recovered donor.

Bioassay for AAI activity. Strains were analyzed for the production of AAI by two methods. The first method was to streak the test strain adjacent to strain A136(pCF240 *traA-lacZ113*)(pCF251) on ATGN agar plus X-Gal. The *lac* fusion of pCF240 *traA-lacZ113* was induced only when AAI was supplied by the adjacent strain. The second method was to add cell-free supernatants (25 μl) of stationary-phase cultures to 3-ml cultures of strain A136(pCF240 *traA-lacZ113*)(pCF251) in ATGNO broth. Supernatants containing AAI resulted in the high-level induction of β-galactosidase activity from the Mu*dJ* fusion.

RESULTS

Identification of a conjugation regulatory gene. An *A. tumefaciens* R10 mutant that constitutively transfers its Ti plasmid was isolated from a library of transposon mutants generated with Tn*5-gusA7*. This mutant, designated KYC6, was originally identified as having octopine-inducible *gus* reporter gene expression (30) and was later found to transfer the Ti plasmid efficiently in the absence of octopine (Table 2). The KYC6 transposon insertion was localized on the Ti plasmid approximately 16 kb to the left of the previously described *occR* gene (Fig. 1) (37).

Various fragments of Ti plasmid DNA including and adjacent to the KYC6 transposon insertion were introduced on multicopy plasmids into a wild-type strain and tested for the ability to stimulate conjugation. pCF212, pCF214, and pCF218 (Fig. 1 and Table 2) stimulated octopine-independent conjugation above uninduced levels, while pCF211 did not. Significantly, pCF218 lacks any Tn*5-gusA7* sequences. Therefore, this plasmid probably does not stimulate conjugation by the same mechanism as the original Tn*5-gusA7* transposon in strain KYC6. The underlying cause of the hyperconjugal phenotype of strain KYC6 remains unclear and is the subject of current studies.

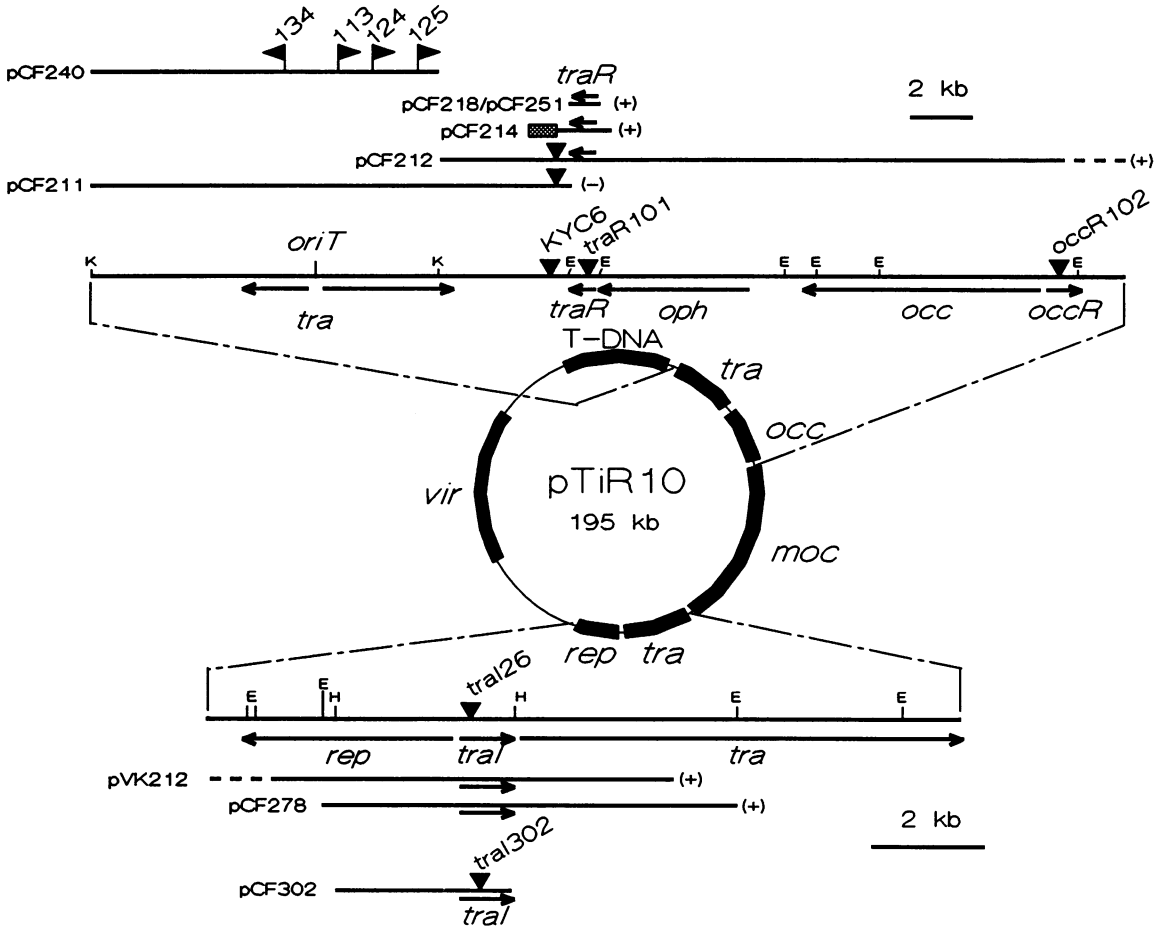

FIG. 1. Localization of the *traR* and *traI* genes. Enlarged regions of the circular map depict the locations of the *traR* and *traI* genes and flanking genes. Clusters of *tra* genes are inferred from heteroduplex studies (28) with regions of the Ti plasmid known to be essential for the conjugal transfer of nopaline-type Ti plasmid pTiC58 (6). The site labeled *oriT* is inferred by sequence similarity (2) with that identified for pTiC58 (12). Thin solid lines represent cloned fragments; (+) or (−) to the right of each line indicates the ability or inability of the construct, respectively, to direct octopine-independent conjugation for *traR* subclones and to synthesize detectable AAI for *traI* subclones. The hatched bar for pCF214 indicates a fragment of the Tn*5-gusA7* $IS50_R$ element. Flags indicate MudJ-derived, TraR-regulated *lacZ* fusions (see the text). Inverted triangles illustrate insertion sites for (i) Tn*5-gusA7* for KYC6 and *traI26*, (ii) *lacZ* fusions *traR101* and *traI302*, and (iii) the *occR102* Ω insertion. Scale bars are provided for each linear map. Restriction sites: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

The *Eco*RI fragment in pCF218 stimulated conjugal transfer only when inserted in one orientation, indicating a requirement for a vector promoter. pCF218 also strongly stimulated conjugal transfer of the widely studied octopine-type Ti plasmid pTiA6, which was previously thought to be conjugation deficient, and of the nopaline-type Ti plasmid pTiC58 (data not shown). A second plasmid containing the same *Eco*RI fragment (Fig. 1) in a different vector (pCF251) also imparted octopine-independent transfer (Table 2), and this was again orientation dependent.

Identification of *traR*. DNA sequencing of the insert carried by pCF218 revealed a single open reading frame of 702 bp preceded by a probable ribosome binding site (62) (Fig. 1). This gene, designated *traR*, encodes a 27.6-kDa predicted protein of 234 amino acids. It contains a potential helix-turn-helix motif (7) in its carboxyl-terminal half. Deletion of the 3′-terminal 66 codons of this gene (pCF234) or introduction of a frameshift mutation at codon 94 (pCF242) abolished its ability to stimulate conjugation (Table 2). DNA sequencing of the region directly upstream of *traR* revealed at least four genes (*oph*; Fig. 1) whose products are homologous to a family of oligopeptide permeases (30). The *traR* promoter sequence has not been identified but is located upstream of the *oph* operon (Fig. 1). Searches of the GenBank-EMBL sequence data base with BLAST (3) showed that TraR is homologous along its entire length to members of the newly described LuxR family of transcriptional regulators (31), with 15 to 25% identity to other members of this family. LuxR-type proteins have been found in several diverse bacterial genera, and there is evidence that members of this family may be widespread in bacteria (4).

The TraR protein from the nopaline-type Ti plasmid pTiC58 was recently characterized (55); the TraR proteins from the nopaline- and octopine-type Ti plasmids share 81% identity (Fig. 2A). Although many of the nonidentities represent conservative substitutions, there are a significant number of differences in the amino-terminal halves of the proteins, regions that might, by analogy to LuxR, interact with autoinducer molecules. The nopaline and octopine *traR* genes share 83% identity across 716 bp of coding sequence. Regions immediately flanking the *traR* coding regions do not show any significant similarity.

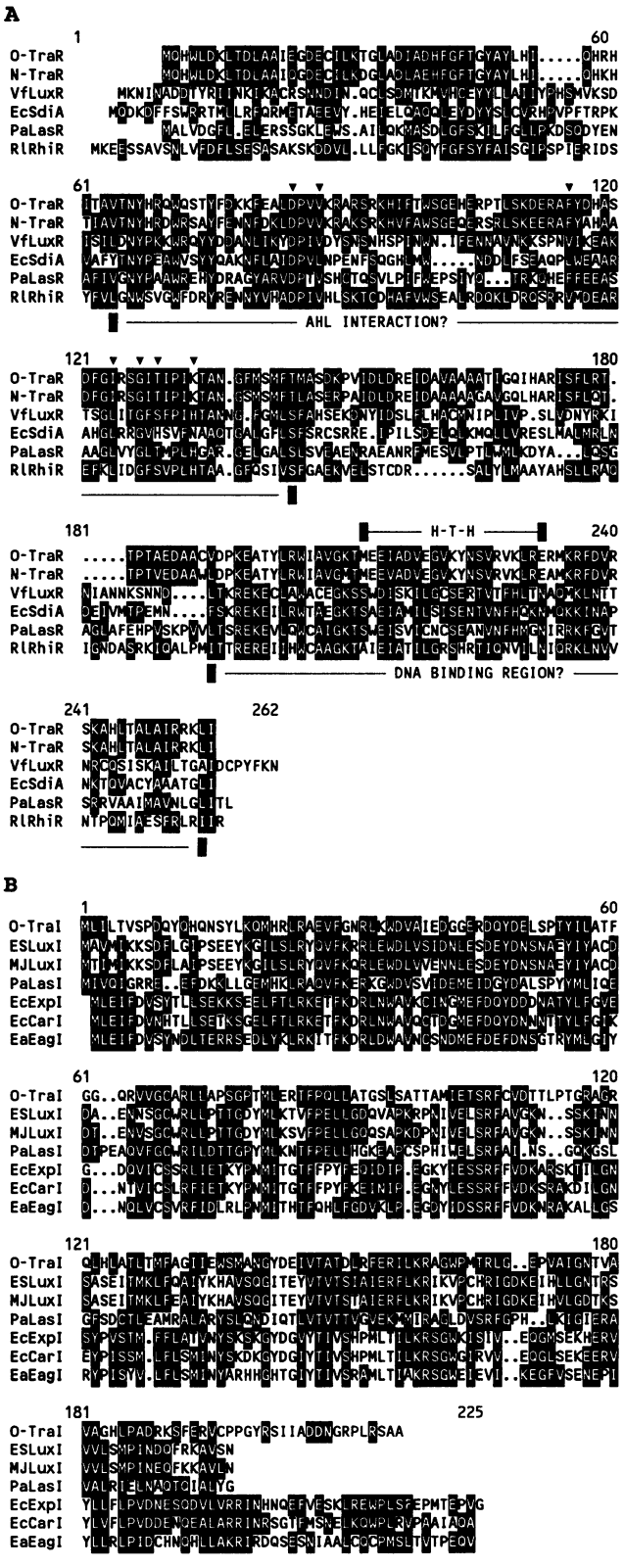

FIG. 2. Alignment of TraR (A) and TraI (B) with homologous proteins. Amino acid sequences were aligned with the PILEUP program of the UWGCG package of sequence analysis programs. Identical residues and conservative changes, as assessed by use of a modified version of the UWGCG Pretty default criteria, are highlighted. Inverted triangles indicate LuxR residues at which mutations affect interactions with VAI (60, 63). AHL, proposed *N*-acyl homoserine lactone interaction region; H-T-H, putative helix-turn-helix motif, as defined for the LuxR protein of *V. fischeri* (31). Homologous sequences are described elsewhere (13, 17, 27, 32, 35, 53, 55, 56, 64a, 69).

TABLE 3. Regulation of a *traR-lacZ* fusion

Strain(plasmid)	Genotype or description	β-Galactosidase sp act (Miller units) in the absence (−) or presence (+) of octopine[a]	
		−	+
R1	*traR-lacZ101*	1.5	14.0
RO1	*traR-lacZ101 occR102*	2.0	2.4
R1(pCF218)	*traR* in multiple copies	15.3	12.6
R1(pSW213)	Vector	1.1	13.8

[a] At 400 μg/ml.

Conjugal transfer requires the *traR* and *occR* genes. To determine whether TraR is required for conjugation and controls the expression of *tra* genes, we generated a *traR* allelic replacement mutant. A *lacZ*-Km^r protein fusion cassette (67) was introduced into the pTiR10 *traR* gene in frame at codon 94 (Fig. 1). Allelic replacement of the resident *traR* gene with this mutation generated simultaneously a *traR-lacZ* fusion and a *traR* null mutation, designated *traR-lacZ101*. This mutation abolished conjugal transfer and was complemented by supplying *traR* on a multicopy plasmid (Table 2).

The *occR* gene (Fig. 1) was disrupted similarly by use of an Sp/Sm Ω cassette (29) to generate *occR102*. The resulting mutant was unable to utilize octopine and was deficient in conjugal transfer (Table 2). Both phenotypes were recessive in a merodiploid strain containing *occR* on a multicopy plasmid. The introduction of *traR* on a multicopy plasmid also suppressed the Tra$^-$ phenotype of the *occR* mutant (but not its Occ$^-$ phenotype), indicating that for conjugation, *traR* in multiple copies is epistatic to an *occR* null mutation.

Both OccR and TraR activate the *traR* gene. The expression of the *traR-lacZ101* fusion (strain R1) was induced about 10-fold by overnight incubation with octopine (Table 3). This induction was abolished by the *occR102* mutation (strain RO1), demonstrating that *traR* induction by octopine requires OccR. These data explain the previous observations that octopine is required for ex planta Ti plasmid conjugation (44) and that *tra* genes are coregulated with *occ* genes (45). Provision of *traR* on a multicopy plasmid elevated β-galactosidase levels from *traR-lacZ101* (Table 3) and caused expression to become octopine independent and OccR independent. These results suggest that TraR may positively regulate its own gene and imply that *traR* may have two promoters, one activated by OccR and the other activated by TraR itself.

TraR activates *tra* genes in the presence of AAI. To facilitate further the analysis of TraR function, we constructed a series of *lac* fusions to conjugal transfer genes that might be regulated by TraR. *Kpn*I fragment 7 of pTiR10 was hypothesized to contain a group of *tra* genes on the basis of its ability to form heteroduplexes with a *tra* region of pTiC58 (6, 28). This fragment was cloned into pSW213 (creating pCF240; Fig. 1) and mutagenized with the Mu*dJ* transposon, generating transcriptional fusions to *lacZ* (9). The insertion derivatives were screened for higher β-galactosidase activity in hyperconjugal strain KYC6 than in strain R10. Several fusions had this

property (Fig. 1), and the expression of each fusion was further induced by octopine in both backgrounds. Strains harboring pCF240 with Mu*dJ* insertion 113 showed the strongest induction, and this fusion was chosen for further analysis. The Mu*dJ* transposon in this mutant was fused to the promoter of a gene we now designate *traA*. The predicted *traA* gene product is homologous to that of the *mobA* gene of plasmid RSF1010 (2). A quantitative analysis of this fusion is provided below.

The *tra*::*lacZ749* fusion in a *tra* region of the nopaline-type Ti plasmid pTiC58 (55) is strongly induced by nopaline-type TraR in the presence of exogenous AAI (74). The nopaline *tra* region forms heteroduplexes with the region flanking the *traA-lacZ113* fusion isolated here (28). We therefore measured the expression of the *traA-lacZ113* fusion in the Ti-plasmidless strain A136 and supplied the *traR* gene on a multicopy plasmid and/or exogenous AAI. In the absence of *traR*, 60 units of β-galactosidase was detected whether or not octopine was present (Fig. 3). Supplying *traR* in multiple copies from pCF251 (*traR* is expressed from a vector promoter and obviates the octopine requirement) actually inhibited expression about threefold, indicating that TraR might repress *traA*. The addition of synthetic AAI to A136(pCF240 *traA-lacZ113*) (pCF251) caused significant β-galactosidase induction (Fig. 3). Again, octopine had no effect on the expression of the *traA-lacZ113* fusion (data not shown). An AAI dose-response curve showed half-maximal induction at roughly 0.2 nM AAI and a decrease in *lacZ* reporter gene expression above 2 nM (Fig. 3). These results indicate that TraR and exogenous AAI are sufficient to activate *traA* expression. Interestingly, a similar dose-response curve generated for the nopaline TraR protein (74) showed half-maximal induction at approximately 1 nM AAI, and this induction was not reduced at higher AAI concentrations. Thus, although TraR-dependent gene activation in the octopine and nopaline systems is likely to be highly similar, there may be subtle differences in the sensitivity to AAI and the magnitude of activation.

The expression of the *traA-lacZ* fusion was also examined with R10(pCF240 *traA-lacZ113*), in which *traR* is provided from the Ti plasmid and expressed from its native promoter(s). AAI alone did not induce *traA-lacZ113* expression, but AAI plus octopine strongly induced β-galactosidase activity in broth cultures (Table 4), presumably because octopine is required to activate the transcription of *traR*. In KYC6, the *traA-lacZ113* fusion was expressed at high basal levels but remained inducible by octopine alone or together with AAI (Table 4), reminiscent of the hyperconjugal phenotype of this strain. No activation of *traA-lacZ113* expression was observed in either the *traR* or the *occR* null mutants (Table 4).

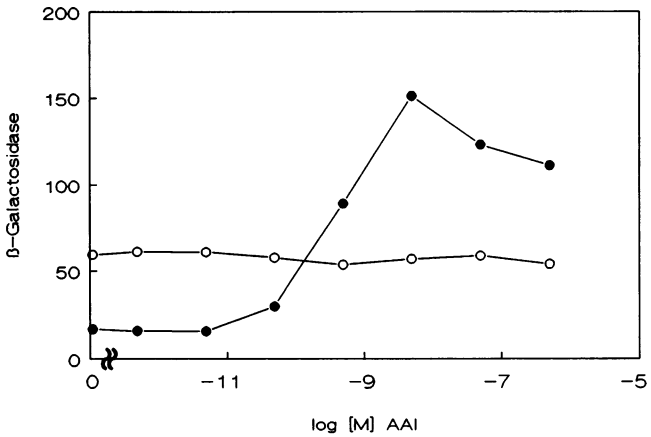

FIG. 3. Dose-response curves for a *traA-lacZ* fusion in a Ti-plasmidless strain. *A. tumefaciens* A136(pCF240 *traA-lacZ113*) harboring either pCF251 (●) or a pDH99 vector control (○) was induced for 12 h in ATGN broth plus a range of AAI concentrations. β-Galactosidase activity from the *lacZ* reporter gene was measured by standard assay protocols (see Materials and Methods), and specific activity was plotted against AAI concentration.

***A. tumefaciens* R10 synthesizes a compound with AAI activity.** We used a derivative of strain A136 harboring the *traA-lacZ113* fusion in a bioassay for the detection of AAI in cell-free culture fluids. A stimulatory compound was present in the supernatants of strain R10 cultured to late exponential phase in the presence of octopine (Table 5). This stimulatory activity was most pronounced in older cultures that were well into stationary phase. The hyperconjugal strains KYC6, R10(pCF218), and R10(pCF251) synthesized high levels of this factor in the presence or absence of octopine, and this synthesis was unaffected by the phase of growth. Null mutations in *traR* or *occR* abolished the production of this stimulatory factor. Introduction of the *traR*-expressing plasmid pCF218 complemented the *traR* null mutant and also suppressed the *occR* null mutant, resulting in a high level of constitutive factor synthesis (Table 5). This stimulatory factor was extracted efficiently into ethyl acetate, a property common to *N*-acyl-homoserine lactones (23). While we do not provide direct chemical evidence that this stimulatory factor is the previously described conjugation factor (74), our genetic and physiological data suggest strongly that the two compounds are identical. Furthermore, AAI was identified originally by use of

TABLE 4. *traA-lacZ113* expression in Ti plasmid-harboring strains

Strain	Genotype or description	β-Galactosidase sp act (Miller units) under the following induction conditions:							
		Liquid				Solid			
		No addition	Octopine[a]	AAI[b]	Both additions	No addition	Octopine[a]	AAI[b]	Both additions
R10	Wild type	164	183	208	1,656	187	1,721	192	3,363
KYC6	Hyperconjugal mutant	1,158	3,363	1,342	3,764	1,600	3,703	1,679	3,548
R1	*traR-lacZ101*	312	205	247	216	217	209	215	212
SR1	*occR102*	213	191	253	284	213	172	210	219
FPA26	*traI26*	87	97	ND[c]	128	113	120	ND	126
FPA26	*traI26*	ND	ND	98	744	ND	ND	126	354

[a] At 2 mg/ml.
[b] Synthetic AAI was used at 0.5 nM for all inductions, except for those in the last row, for which it was used at 5 nM.
[c] ND, not determined.

TABLE 5. Production of AAI activity by *Agrobacterium* strains

Strain(plasmid)	Genotype or description	AAI activity[a] in the absence (−) or presence (+) of octopine[b]	
		−	+
R10	Wild type	−	+
KYC6	Hyperconjugal mutant	+	+
R10(pCF218)	*traR* (multiple copies)	+	+
R10(pCF251)	*traR* (multiple copies)	+	+
R1	*traR-lacZ101*	−	−
R1(pCF218)	*traR-lacZ101 traR* (multiple copies)	+	+
SR1	*occR102*	−	−
SR1(pCF218)	*occR102 traR* (multiple copies)	+	+
FPA26	*traI26*	−	−
FPA26(pCF278)	*traI26 traI* (multiple copies)	−	+

[a] The AAI bioassay was carried out as described in Materials and Methods. +, induction of β-galactosidase reporter gene expression at least equal to that in a 0.2 nM AAI positive control; −, no induction.

[b] Source cultures were grown in ATO broth; octopine was used at 2 mg/ml.

an octopine-type Ti plasmid (73). We will therefore refer to this stimulatory factor as AAI.

Identification of the putative synthase gene, *traI*. To identify the gene(s) required for AAI synthesis, we screened an ordered cosmid library (46) constructed from the octopine-type Ti plasmid pTiA6NC (a plasmid virtually identical to pTiR10). We first determined that cell-free culture fluids of *A. tumefaciens* harboring pTiA6NC and *traR* carried on pCF251 produced high levels of AAI activity, as determined by our bioassay (data not shown). A number of the ordered cosmids, chosen to span the entire Ti plasmid, were introduced into strain C58 C1RS(pCF251), which lacks a Ti plasmid and contains *traR* on a multicopy plasmid. Culture supernatants of the resulting strains were bioassayed for AAI. C58 C1RS(pCF251) harboring any of the overlapping cosmids pVK211, pVK212, pVK213, and pVK214 was strongly positive in the bioassay. Strains carrying these cosmids alone (i.e., in the absence of *traR*) also produced low but detectable levels of AAI. All AAI-producing cosmids contained a region of the Ti plasmid adjacent to the replication (*rep*) operon (Fig. 1) (65).

Two plasmids carrying the *rep* operon and flanking sequences (pCF278 and pCF302; Fig. 1) directed AAI synthesis in the presence of *traR* in *A. tumefaciens*. Plasmid pCF278 also directed the synthesis of detectable levels of AAI in *E. coli* (data not shown); again, this synthesis was dependent on *traR* carried on pCF251. These data strongly suggested that the AAI synthase gene(s) was adjacent to *rep*. DNA sequencing of this region revealed an open reading frame of 212 codons with a probable translation start site 303 bp counterclockwise from *rep* (Fig. 1). The predicted 23.4-kDa protein, designated TraI, is homologous over its entire length to the *luxI* gene product of *V. fischeri* (30.6% identity; 67.3% similarity) and to a number of other LuxI homologs (Fig. 2B).

Genetic analysis of *traI*. A mutant carrying a transposon insertion in *traI*, designated FPA26, was generated by use of Tn*5-gusA7* by screening R10 insertion derivatives for β-glucuronidase induction on octopine-supplemented solid media, specifically when grown adjacent to an AAI-producing strain. Sequencing of DNA flanking the Tn*5-gusA7* insertion site in FPA26 revealed that the transposition had occurred at codon 18 of the *traI* gene. Streaking of FPA26 adjacent to the constitutive AAI producer KYC6 caused strong β-glucuronidase activity in areas in which the two strains were closely juxtaposed, while culturing of FPA26 adjacent to a *traR* null mutant (strain R1) did not cause induction. FPA26 did not secrete detectable AAI under any conditions tested, and this defect was complemented by providing *traI* in *trans* [Table 5; strain FPA26(pCF278)]. In broth cultures, the induction of β-glucuronidase activity required octopine plus unusually high levels of cell-free culture fluids from the AAI-overproducing strain KYC6 (Table 6), indicating that *traI* is activated by TraR-AAI complexes, potentially creating a positive-feedback loop similar to that seen in the *V. fischeri lux* system (26). Similar results were obtained when synthetic AAI instead of cell-free culture fluids was used (data not shown). Likewise, the *traA-lacZ113* fusion introduced into FPA26 (on pCF240) was not induced by octopine or synthetic AAI alone but was induced in the presence of octopine and high levels of synthetic AAI (Table 4). The fact that low levels of AAI did not induce this fusion in FPA26 but did induce the same fusion in R10 suggests that *tra* gene activation by low levels of AAI in wild-type R10 might be amplified by expression of the resident *traI* gene.

FPA26 was deficient in conjugal transfer (Table 2). This deficiency was not rescued by pCF278 (a plasmid that complements the mutation for AAI synthesis) or when exogenous AAI was provided. This result raises the possibility that the Tn*5-gusA7* insertion is polar for the expression of downstream genes required for conjugal transfer (Fig. 1). This observation is corroborated by DNA sequence analysis (2). Therefore, the expression of downstream genes probably originates from a promoter upstream of *traI* and is activated by TraR and AAI. Because *traI* is adjacent to and expressed divergently from the *rep* operon, it is likely to be the first gene of an operon of *tra* genes. Such a positioning of *traI* at the beginning of an operon is reminiscent of the *lux* operon, in which *luxI* is the first gene.

High level-TraR-TraI-dependent induction occurs only on solid media. When harbored in wild-type strain R10, the *traA-lacZ113* fusion carried on pCF240 was strongly induced in β-galactosidase assays when exogenous AAI and octopine were supplied (Table 4). However, induction of the fusion was not observed when octopine alone was supplied, whether the cells were in exponential phase or stationary phase. This result was surprising because *A. tumefaciens* R10 has a functional *traI* gene and can synthesize AAI in amounts detectable by our standard AAI bioassay. Similarly, poor induction was also observed for the *cis* merodiploid R10PI1, in which the *traI-lacZ302* fusion and the wild-type *traI* gene are both present in single copies, thus excluding the possibility that the poor induction of *traA-lacZ113* carried on pCF240 is due to a multicopy effect (Table 6). These results were inconsistent with both the AAI bioassays and the conjugal transfer efficiencies for strains carrying wild-type pTiR10, for which octopine alone was sufficient to stimulate AAI synthesis and conjugal transfer (Tables 2 and 5). Therefore, we repeated octopine induction experiments identical to those discussed above, except that the strains were induced on 0.2-μm-pore-size filters on solid media (1.5% agar), as in the conjugal transfer assays. Interestingly, β-galactosidase assays of strains grown on filters revealed high levels of *traA-lacZ113* (Table 4) and *traI-lacZ302* (Table 6) induction in the presence of octopine alone. As expected, induction of *traA-lacZ113* in the *traR* and *occR* null mutants was not observed under any conditions (Table 4). The discrepancy in *tra* gene induction between liquid and solid media containing octopine alone was abolished by the addition of exogenous AAI (Tables 4 and 6). Moreover, β-glucuronidase activity from the *traI-gusA7* fusion in the *traI* null mutant

TABLE 6. *traI* fusion expression in Ti plasmid-harboring strains

Activity	Strain(plasmid)	Genotype	Reporter gene product sp act (Miller units) under the following induction conditions:							
			Liquid				Solid			
			No addition	Octopine[a]	AAI[b]	Both additions	No addition	Octopine[a]	AAI[b]	Both additions
β-Glucuronidase	FPA26	*traI26*	3	3	3	30	3	4	4	31
	FPA26(pCF278)	*traI-gusA26 traI* (multiple copies)	15	13	17	181	31	67	36	100
β-Galactosidase	R10PI1[c]	*traI-lacZ302 traI*	18	14	18	1,203	103	1,834	112	2,494

[a] At 2 mg/ml.
[b] In 25 μl of KYC6 cell-free culture fluid (10 times the amount needed to induce *traA113-lacZ* in wild-type R10).
[c] *cis* merodiploid.

FPA26 was not increased by incubation on solid media (Table 6). It should be noted that the expression of the *traA-lacZ113* fusion in the constitutive mutant KYC6(pCF240) was not dramatically affected by induction on solid media (Table 4). We conclude that the activation of *tra* genes in octopine-type *Agrobacterium* strains requires a factor(s) or parameter(s) specific for solid-phase growth and that the effect of this factor(s) is manifested through the TraR-TraI-AAI regulatory circuit.

DISCUSSION

The initiation of conjugal transfer in *A. tumefaciens* is strictly regulated by tumor-released opines (54) and, for octopine-type Ti plasmids, the conjugal opine is octopine itself. The induction of *occ* genes by octopine requires the LysR-type regulator OccR (37, 68). We show here that OccR is required to activate *traR* and that this is the underlying mechanism by which octopine activates conjugal transfer. Given the similarities between the TraR and LuxR regulators and their *N*-acyl homoserine lactone inducers, it was plausible that the AAI synthase gene would be homologous to *luxI*. We have now determined that this gene, denoted *traI*, is 30.6% identical to *luxI* at the amino acid level (Fig. 2B) and is encoded divergently from the *rep* operon of the Ti plasmid (Fig. 1). Analysis of the sequences upstream of *traA* and *traI* revealed nearly identical 18-bp imperfect inverted repeats (for *traA*, ATGTG CACATCTGCACAT; for *traI*, ACGTGCAGATCTGCA CAT). Interestingly, a third copy of this sequence is located further upstream of *traI* (AAGTGCAGATTTGCACAT), proximal to the divergent *rep* operon. These three inverted repeats, tentatively designated *tra* boxes (31), are strikingly similar to two 19-bp LuxR operator sequences (17, 59). We speculate that these sequences may provide binding sites for TraR.

Model for the regulation of Ti plasmid conjugal transfer in *Agrobacterium* strains. Collectively, the data presented here allow us to formulate a model for the regulation of Ti plasmid conjugal transfer (Fig. 4). As in the LuxR-LuxI system, the activation of TraR-AAI-regulated target genes requires an increase in the pool size of either TraR or AAI or both. In octopine-type *A. tumefaciens*, this is accomplished by placing *traR* under the control of OccR, causing the entire process to be octopine responsive. Our results suggest that OccR-octopine complexes directly activate the transcription of *traR*. Because constitutive expression of *traR* from nonnative promoters bypasses the requirement for octopine, we believe that this is the primary point of control for the opine-mediated induction of conjugal transfer. The *traI* gene product directs basal-level synthesis of AAI from precursors present in the cell, and AAI, by analogy to VAI, is able to freely permeate the bacterial envelope. At some threshold concentration, TraR interacts with AAI to activate the transcription of *tra* genes. We hypothesize that TraR-AAI complexes bind regions upstream of *tra* genes. The inverted repeat located between divergently oriented, TraR-regulated promoters at *oriT*, as well as similar repeats found upstream of *traI*, are likely candidates for TraR binding sites. Among the *tra* genes activated by TraR-AAI are *traI* and perhaps *traR* itself. This positive feedback probably ensures that once the appropriate conditions are attained, conjugation is strongly activated. This autoamplification of *traI* and *traR* expression is reminiscent of the LuxR-LuxI system.

Although the *occR*, *traR*, and *traI* gene products are key components in the regulation of Ti plasmid conjugal transfer, other genes may also be involved in this control. For instance, the KYC6 transposon insertion causes a dramatic increase in the level of Ti plasmid conjugal transfer (Table 2), *tra* gene activation (Table 4), and AAI synthesis (Table 5) in the presence or absence of octopine. This insertion lies in a small open reading frame oriented convergently to *traR* (30). The mechanism by which the KYC6 mutant has been uncoupled from octopine regulation is unknown, but this uncoupling suggests that additional factors may impinge upon TraR-AAI-mediated gene activation.

For the broad-host-range octopine-type Ti plasmids, there are at least two interesting parallels between the regulation of *tra* genes and the regulation of *vir* genes, which mediate the transfer of T-DNAs into plant cell nuclei. First, both systems are responsive to chemical signals released from plant hosts, as *tra* genes are induced by opines and *vir* genes are induced by a combination of phenolic compounds, acid pH, and monosaccharides that are found at plant wound sites (71). Second, both systems contain positive autoregulatory loops, since putative TraR-AAI complexes activate both *traR* and *traI*, while the *vir* gene activator VirG activates the *virG* gene (64). In both cases, the effect may be to create a transcriptional switch which can be readily maintained in either a fully inactive state or a fully active state.

What is the function of TraR-mediated regulation? The LuxR-LuxI system of *V. fischeri* is thought to enable cells to monitor their own density and activate *lux* genes specifically at high densities (22). By analogy to the *V. fischeri* system, TraR, TraI, and AAI may facilitate intercellular communication between *A. tumefaciens* donor cells and allow the coordination of conjugal transfer of the Ti plasmid at high cell densities. Our observation that *tra* gene induction is significantly stronger on solid media agrees with that of Zhang and Kerr (73), who

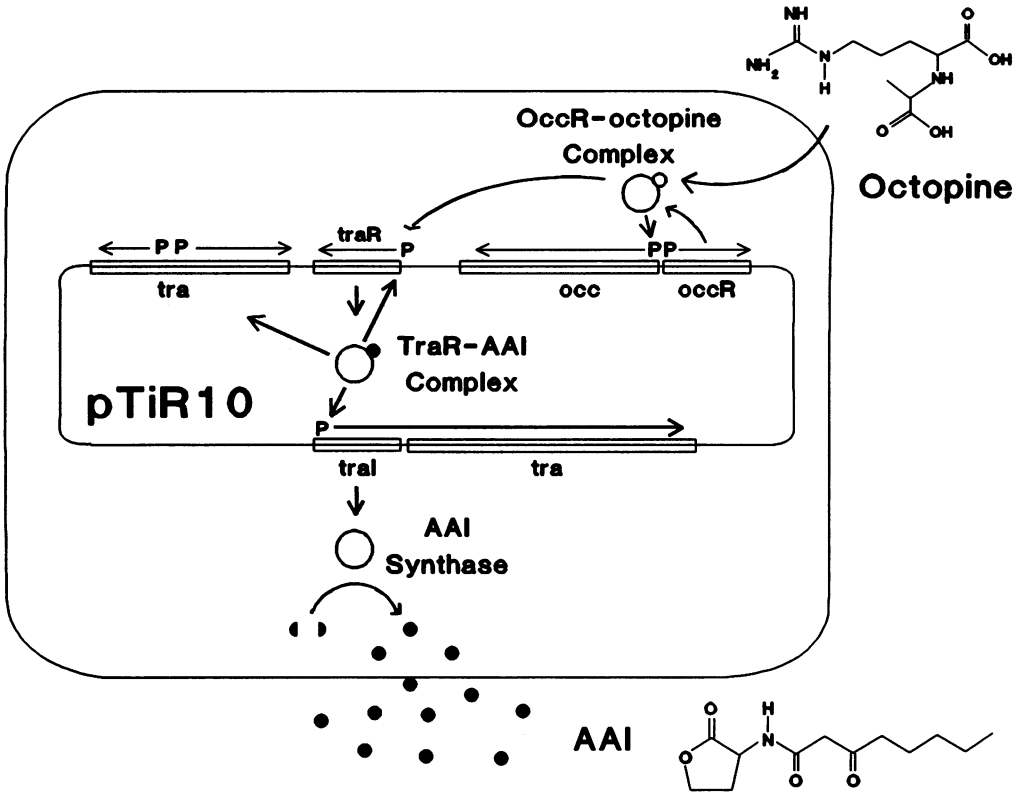

FIG. 4. Tentative model for *Agrobacterium tra* gene regulation. P, promoter.

observed that the synthesis of bioactive AAI in wild-type strains was augmented strongly by growth on solid media. It is difficult to obtain a cell density and cell-to-cell proximity in a liquid culture equivalent to that found in a solid-phase culture; thus, the differences observed between liquid and solid cultures may reflect a cell density requirement similar to that defined for the LuxR-LuxI system (31, 52). However, there may be other factors specific to a solid culture that are crucial for *tra* gene induction, such as diffusion barriers, cell-cell contact, and oxygen limitation. Because the addition of synthetic AAI or cell-free culture fluids from AAI-producing strains abolished the effect of a solid culture, we conclude that these factors must be sensed through the TraR-AAI signal transduction pathway. AAI is synthesized exclusively from Ti plasmid-harboring cells; thus, the ability to perceive cell density and/or other factors would be donor cell specific.

Why is conjugal transfer of the Ti plasmid controlled by autoinduction? It is far from obvious what benefit is gained from regulating conjugation in a donor cell-dependent fashion. This system differs from the *E. facaelis* conjugation system, in which donor cells sense a pheromone released from recipient cells (11). Perhaps *A. tumefaciens* uses the donor-synthesized pheromone to gauge the overall bacterial population in or around a tumor. Alternatively, it is possible that conjugation occurs between donor cells. For tumors harboring different strains of *A. tumefaciens*, such a transfer would increase the spectrum of opines utilized by each strain and might also increase the gene dosage of opine catabolic genes. However, conjugation between two plasmid-containing cells is normally prevented by entry exclusion systems (70). The possibility also exists that the purpose of the TraR-AAI system is to serve as a signal amplification system within each bacterium as well as to serve as (or instead of) a signal disseminator between bacteria.

Comparison of the octopine and nopaline signal cascades. It was not surprising that the nopaline and octopine TraR proteins are so similar, since the Tra regions of octopine-type and nopaline-type plasmids form stable heteroduplexes observable by electron microscopy (14, 28, 40). Conjugation in both types of plasmids is controlled by opines, and strains carrying both types of plasmids produce diffusible molecules with similar activities (73, 74). However, the mechanisms by which opines control conjugation are strikingly different in the two types of plasmids. As described above, octopine-type Ti plasmid *traR* is activated by OccR, a LysR-type transcriptional activator (37, 68). Null mutations in *occR* prevent octopine catabolism, conjugation, *traR* induction, and AAI synthesis. In contrast, nopaline-type Ti plasmid *traR* is probably under the negative control of the AccR repressor, and repression is relieved by agrocinopine or by null mutations in *accR* (5). OccR is not homologous to AccR. The fact that the two *traR* genes are regulated in such different ways indicates that there is strong selection to maintain opine regulation of *tra* genes and that this hierarchy has evolved at least twice, a remarkable example of convergent evolution.

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