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Quorum-Dependent Mannopine-Inducible Conjugative Transfer of an *Agrobacterium* Opine-Catabolic Plasmid

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The Ti plasmid in *Agrobacterium tumefaciens* strain 15955 carries two alleles of *traR* that regulate conjugative transfer. The first is a functional allele, called *traR*, that is transcriptionally induced by the opine octopine. The second, *trlR*, is a nonfunctional, dominant-negative mutant located in an operon that is inducible by the opine mannopine (MOP). Based on these findings, we predicted that there exist wild-type agrobacterial strains harboring plasmids in which MOP induces a functional *traR* and, hence, conjugation. We analyzed 11 MOP-utilizing field isolates and found five where MOP induced transfer of the MOP-catabolic element and increased production of the acyl-homoserine lactone (acyl-HSL) quormone. The transmissible elements in these five strains represent a set of highly related plasmids. Sequence analysis of one such plasmid, pAoF64/95, revealed that the 176-kb element is not a Ti plasmid but carries genes for catabolism of MOP, mannopinic acid (MOA), agropinic acid (AGA), and the agrocinopines. The plasmid additionally carries all of the genes required for conjugative transfer, including the regulatory genes *traR*, *traI*, and *traM*. The *traR* gene, however, is not located in the MOP catabolism region. The gene, instead, is monocistronic and located within the *tra-trb-rep* gene cluster. A *traR* mutant failed to transfer the plasmid and produced little to no quormone even when grown with MOP, indicating that $TraR_{pAoF64/95}$ is the activator of the *tra* regulon. A *traM* mutant was constitutive for transfer and acyl-HSL production, indicating that the anti-activator function of TraM is conserved.

Ti plasmids, found in members of the genus *Agrobacterium*, encode two conjugative-transfer systems, both of which are regulated at the transcriptional level in response to compounds produced by the host plant. The *vir* system, responsible for transfer of the T strand of the Ti plasmid to plant cells, is induced by, among other factors, phenolic compounds produced by wounded plant cells (reviewed in references 1, 2, and 3). The transferred strand is subsequently integrated into the plant cell nuclear genome (reviewed in reference 4) and results in production of plant growth hormones responsible for the crown gall tumors (reviewed in reference 5). Additionally, the integrated transfer DNA (T-DNA) carries the genes for synthesis by the neoplasias of novel compounds called opines (reviewed in references 5 and 6). Opines are specific conjugates of sugars or, more frequently, amino acids and α-ketoacids or sugars, and they serve as carbon and sometimes as nitrogen or phosphorus sources for the bacteria that induced the tumor (reviewed in reference 6). Remarkably, the Ti plasmid also carries the genes for uptake and catabolism by the bacteria of the specific opines produced by the tumor. The second transfer system, encoded by the *tra* regulon, is induced by certain opines that are produced by the tumors induced by the bacterium. This system is responsible for conjugative transfer of the plasmid from one bacterium to another (7, 8).

Ti plasmids generally code for uptake and catabolism of two or three of the eight known opine families (7, 9–11). However, in the vast majority of such *Agrobacterium* megaplasmids studied to date, only one opine type induces transfer and thus is dubbed the conjugative opine (7). For instance, pTiC58 codes for the uptake and catabolism of two opine types, nopaline and agrocinopines A and B, but only the agrocinopines induce plasmid transfer (12). The conjugative opines, however, do not directly induce transfer of pTiC58; rather, they control the transcription of *traR*, which codes for the quorum-sensing (QS) activator TraR (13, 14). TraR directly activates the Ti plasmid *tra* and *trb* operons, responsible for DNA metabolism and mating-pair formation, respectively (15–20).

In the studied systems, the link between the opine signal and the quorum-sensing system results from the inclusion of *traR* in an operon whose expression is itself regulated by the conjugative opine. For example, *traR* of pTiC58 is located in the *arc* operon, which is adjacent and in opposite orientation to the agrocinopine A and B uptake and catabolism operon (*acc*) (14). The divergently oriented *acc* and *arc* operons are both transcriptionally repressed by the opine-responsive repressor AccR (13, 14). When the agrocinopines are available, repression by AccR is lifted, and *traR*, as part of the *arc* operon, is expressed (14). Opine-mediated expression of TraR, however, is not sufficient for induction of conjugative transfer. The active form of TraR requires its ligand, *N*-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL), the *Agrobacterium* autoinducer (AAI) (21–23). This acyl-HSL quormone is synthesized by TraI, which is encoded by the first gene of the Ti plasmid *trb* operon (18, 24). QS-dependent induction of transfer is additionally modulated by TraM, an antiactivator that interacts with TraR, thereby inhibiting activation of the *tra* regulon (25–27).

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This article is dedicated to the late Larry Moore of Oregon State University.

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Although in most cases only one opine serves as the conjugative signal, this is not always the case. For instance, *Agrobacterium radiobacter* strain K84 harbors an opine-catabolic megaplasmid, pAtK84b, that is inducible for transfer by two opine types, agrocinopines A and B and nopaline (12, 28). The plasmid carries two copies of *traR*, and each is independently transcribed. One, $traR_{noc}$, is in an operon induced by nopaline, while the other, $traR_{acc}$, is in an operon similar to *arc* of pTiC58 and is inducible by agrocinopines A and B (28). The two alleles function independently. For example, in a $traR_{noc}$ mutant, conjugative transfer is still inducible by agrocinopines. Likewise, if $traR_{acc}$ is mutated, conjugative transfer remains inducible by nopaline, but not by the agrocinopines (28).

The octopine-mannityl opine-type Ti plasmids, pTi15955 and pTiR10, provide another example of opine-inducible conjugative transfer. Tumors induced by strains harboring these Ti plasmids produce two opine types, octopine and the mannityl opines. In turn, the bacteria can catabolize octopine and all four of the mannityl opines: agropine (AGR), mannopine (MOP), mannopinic acid (MOA), and agropinic acid (AGA) (reviewed in reference 7). These plasmids carry two alleles of *traR*. One, $traR_{oct}$, is a member of an operon that is activated by octopine, and this opine induces conjugative transfer (29). The second, called *trlR*, is the terminal gene in the MOP transport operon, *motABCDtrlR*, located in the mannityl opine-catabolic region of these Ti plasmids (30). Expression of this operon, including *trlR*, is inducible by MOP, but not by octopine (30, 31). Importantly, the *trlR* gene is a dominant-negative frame-shifted mutant of *traR* and encodes a protein with functional N-terminal dimerization and AAI-binding domains, but not the C-terminal DNA-binding domain (30, 31). The protein product can dimerize with the full-size, functional TraR, induced by octopine, thereby inhibiting octopine-inducible conjugative transfer (30–32).

Based on the presence of *trlR* in an operon inducible by MOP, we hypothesized that there exist in nature *Agrobacterium* plasmids in which conjugative transfer is induced by this mannityl opine. We also hypothesized that in such plasmids, the functional *traR* gene would be associated with the *mot* operon. In this study, while we provide evidence concerning a family of plasmids that supports the first hypothesis, the allele of *traR* associated with quorum-dependent transfer is not associated with a *mot*-like operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The mannopine-utilizing field isolates of *Agrobacterium* spp. were obtained from the laboratory of the late Larry Moore, Oregon State University, Corvallis, OR, and have not been previously described in the literature. The methods for collection of the field tumors, isolation of bacteria, determination of opine catabolism, and pathogenicity testing were all described previously (33).

Media and growth conditions. Strains of *Escherichia coli* were grown in L broth (Fischer Scientific) and in SOB or SOC medium (34). Strains of *Agrobacterium* spp. were grown in L broth (Fisher Scientific) or MG/L (35) or on Nutrient Agar (Difco). Stonier's medium (36) was used for agrocin 84 sensitivity assays, and AB (35) and AT (37) media were used as minimal media. Liquid AB medium was supplemented with 0.005% yeast extract (Difco) unless otherwise noted. Minimal media were supplemented with mannitol to 0.2% or with mannopine or mannopinic acid at 500 μg/ml as a carbon source. Mannopine and mannopinic acid were the kind gifts of Yves Dessaux (Institut des Sciences du Végétal, Gif-sur-Yvette, France). Antibiotics were used at the following concentrations (μg/ml): rifampin, 50 or 100; streptomycin, 100; kanamycin, 25 or 50; ampicillin, 100; carbenicillin, 50 or 100; and gentamicin, 25. Cultures of *E. coli* were incubated at 30 or 37°C, and cultures of *Agrobacterium* spp. were incubated at 28°C.

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference[b]
Agrobacterium sp.		
AF1/95	Wild-type mannopine-utilizing strain	L. Moore
AR11N/71	Wild-type mannopine-utilizing strain	L. Moore
B24/93	Wild-type mannopine-utilizing strain	L. Moore
B26/94	Wild-type mannopine-utilizing strain	L. Moore
B98/94	Wild-type mannopine-utilizing strain	L. Moore
B155/93	Wild-type mannopine-utilizing strain	L. Moore
F64/95	Wild-type mannopine-utilizing strain	L. Moore
F265/93	Wild-type mannopine-utilizing strain	L. Moore
J62/95	Wild-type mannopine-utilizing strain	L. Moore
J84/95	Wild-type mannopine-utilizing strain	L. Moore
M200/94	Wild-type mannopine-utilizing strain	L. Moore
15955	Wild-type octopine strain; MOP^+ MOA^+ AGR^+ AGA^+	OC
C58	Wild-type nopaline strain; pAtC58 pTiC58	OC
C58C1RS	Ti plasmidless derivative of C58; pAtC58 Sm^r Rif^r	OC
NTL4	Ti plasmidless derivative of C58; pAtC58 Δ*tetAR*	98
NTL6	Plasmidless derivative of NTL4	OC
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
LE392	*hsdR514*(r_k^- m_k^+) *glnV*(*supE44*) *tryT*(*supF58*) *lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	Promega
S17-1 λpir	Tp^r, Sm^r *recA thi pro hsdR-M*$^+$ RP4::2-Tc::Mu::Km Tn*7* λpir	99
Plasmids		
pAgK84-A1	Agrocin 84 producer	100
pArA4	MOP^+ MOA^+ AGA^+	J. Tempé
pCP13/B	Deletion derivative of pCP13; Tc^r	62
pKD46	Amp^r; λ Red helper plasmid	63
pSRKGm	Gm^r; IPTG-inducible expression vector	66
pViK107	Kan^r; promoterless *lacZY*; *pir* dependent	65
pWM91	Amp^r *sacB*	64
pZLR4	Gm^r; indicator for detection of acyl-HSL	44

[a] $TraI^+$, production of AAI; Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.
[b] OC, our collection.

Biovar determination. The biovar assignments of the wild-type strains were provided to us by the Moore laboratory. We verified these assignments for strains F64/95, F265/93, J62/95, J84/95, and M200/94 using three tests described by Moore et al. (38): 3-ketolactose production, growth on AB agar with erythritol as the primary carbon source, and growth on Nutrient Agar supplemented with 0.25% glucose and 2% NaCl.

Virulence assays. The Moore laboratory described the virulence properties of all wild-type strains as tested on tomato plants (Table 2). We repeated these assays with the five MOP-inducible strains using the stem-wound inoculation method on tomato plants, essentially as described previously (39, 40).

TABLE 2 Characteristics of MOP-utilizing wild-type isolates

Strain[a]	Opine(s) utilized[b]	Conjugation frequency[c]		Isolation		Tumorigenic	Agrocin sensitivity	Biovar
		Uninduced	Induced with MOP	Host	Location			
AF1/95	MOP, OCT	$<10^{-8}$	$<10^{-8}$	Lilac	Visalia, CA	Yes	No	2
AR11N/71	MOP, NOP, OCT	$<10^{-8}$	$<10^{-8}$	Apple	Canby, OR	Yes	ND[d]	ND
B24/93	MOP	3.3×10^{-6}	9.0×10^{-7}	Quince	Portland, OR	No	No	2
B26/94	MOP, NOP	$<10^{-8}$	$<10^{-8}$	Walnut	Portland, OR	Yes	No	2
B98/94	MOP	1.5×10^{-5}	2.6×10^{-6}	Walnut	Gridley, CA	Yes	No	2
B155/93	MOP, NOP	$<10^{-8}$	$<10^{-8}$	Quince	Portland, OR	No	Yes	ND
F64/95	MOP	$<10^{-8}$	6.6×10^{-5}	Apple	Modesto, CA	No	Yes	2
F265/93	MOP	$<10^{-8}$	4.4×10^{-5}	Apple	Yamhill, OR	No	Yes	2
J62/95	MOP, NOP	$<10^{-8}$	4.6×10^{-5}	Apple	Modesto, CA	Yes	Yes	2
J84/95	MOP, NOP	$<10^{-8}$	3.1×10^{-5}	Apple	Modesto, CA	Yes	Yes	2
M200/94	MOP	$<10^{-8}$	3.6×10^{-5}	Apple	Woodburn, OR	No	ND	2

[a] The wild-type isolates listed all utilize mannopine and were obtained from Larry Moore, Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR.
[b] NOP, nopaline; OCT, octopine.
[c] Conjugation frequency was measured as the number of transconjugates recovered per input donor cell.
[d] ND, not determined.

Monitoring growth. Strains F64/95 and NTL6(pAoF64/95) were precultured by growth in LB. In the morning, the cells were collected by centrifugation, washed three times with 0.9% NaCl, and suspended in 1 ml of sterile 0.9% NaCl. The washed cells were inoculated into liquid AB mannitol (ABM), AB plus MOP, or AB with no additional carbon source to a population density of about 10^7 cells/ml. Strains were monitored for growth by turbidity as measured with a Klett colorimeter using a red filter. Periodically, volumes were removed, part of which was frozen at −20°C and used later for AAI detection and quantification. The remainder was immediately used for conjugative-transfer assays and viable-cell determinations.

Conjugative-transfer efficiency. Conjugative transfer of the *Agrobacterium* plasmids to strain C58C1RS (Table 1) was determined using the drop plate method (8, 41, 42). Transconjugants were selected for using opine catabolism or resistance to an appropriate antibiotic, and donors were counterselected using rifampin and streptomycin.

Detection and quantification of AAI. AAI was extracted from the culture supernatant essentially as described previously (8, 43, 44). For chromatographic analysis, the concentrated samples were spotted on a C_{18} reversed-phase thin-layer chromatography (TLC) plate and separated using methanol-H_2O (60:40 [vol/vol]) (8, 44). For quantification, 5-μl volumes of a 2-fold dilution series of each sample were spotted in a grid pattern onto a TLC plate. The samples were allowed to dry, and the plates were overlaid with 1% agar containing 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the acyl-HSL indicator strain NTL4(pZLR4) (8, 44) and incubated overnight at 28°C. The plates were dried and scanned, and ImageJ (version 1.44o; National Institutes of Health [http://imageJ.nih.gov/ij]) (45) was used to measure the area of the blue spots. These values were compared to those of a dilution series of an AAI standard (Sigma) spotted on the same plate (8).

Agrocin 84 sensitivity assays. A modified version of the procedure of Hayman and Farrand (46) was used to assess sensitivity to agrocin 84. Briefly, two colonies of the agrocin 84 producer strain, NT1(pAgK84-A1) (Table 1), were suspended in 0.25 ml of 0.9% NaCl, and 15 μl of the suspension was spotted onto the centers of Stonier's agar (1.5% agar) plates (36) and incubated at 28°C for 3 days. The producer strain was killed by exposure of the plate to chloroform vapor for 15 min. The cultures to be tested for sensitivity were inoculated into MG/L for overnight growth. Two or three drops of these overnight cultures were mixed with 3 ml of melted 0.7% agar and overlaid onto the plates. The cultures were incubated at 28°C, and zones of growth inhibition were assessed daily.

Isolation of mannityl opines from tumors. Crude preparations of the mannityl opines were isolated from plant tumors as described previously (10, 47). Briefly, tomato tumors induced by *Agrobacterium tumefaciens* strain 15955 were minced and placed in 50-ml sterile conical tubes. Enough Milli Q water was added to cover the tops of the minced tissue, and the tubes were placed in boiling water for 10 min. The mixture was ground with a mortar and pestle, and the solids were removed by decanting the mixture over cheesecloth. The remaining particulate matter was removed by centrifugation, and the tumor extract was sterilized by passage through a 0.22-μm filter.

Separation and detection of mannityl opines. Samples containing mannityl opines were spotted on a pencil line drawn across the middle of a sheet of Whatman 3MM paper and allowed to dry. The opines were separated by high-voltage paper electrophoresis (HVPE), using an acetic acid-formic acid buffer with a pH between 1.7 and 1.9 according to methods described previously (10, 39, 48). The opines were visualized using an alkaline silver nitrate reagent, as described previously (35, 39, 49).

Mannityl opine utilization studies. Growth on solid medium was assessed by visual inspection of strains inoculated on AB agar medium with MOP or MOA as the sole source of carbon. The plates were observed daily over a 7-day period. Growth was compared to appropriate positive and negative controls and recorded as follows: −, no growth; +/−, very poor growth; +, good growth; and ++, very good growth. Growth in liquid medium was assessed turbidimetrically as described above. We additionally assessed mannityl opine utilization by the disappearance of opines from liquid culture medium (50). Sterilized tumor extract prepared as described above was added to 2× AT medium in a 1:1 volume ratio. Volumes of 0.5 ml were inoculated with a single colony of the strain to be tested or left uninoculated. The cultures were incubated at 28°C with shaking for 5 days, at which time the cells were removed by centrifugation. A 6-μl volume of each of the culture supernatants was spotted onto Whatman paper and analyzed for the disappearance of mannityl opines by high-voltage paper electrophoresis, as described above.

MOP cyclase activity. Five-microliter volumes of ABM were inoculated with a single colony of the strain to be tested and allowed to grow overnight with aeration at 28°C. Subsequently, MOP was added to the cultures at a concentration of 20 μg/ml. After incubation for an additional 3 h, the cells were harvested by centrifugation, and MOP cyclase activity was determined by the conversion of MOP to agropine as assessed by high-voltage paper electrophoresis, as previously described (51).

Preparation and purification of plasmid DNA. Small-scale plasmid isolation from *E. coli* was done by the alkaline lysis methods of Sambrook and Russell (52). Large *Agrobacterium* plasmids to be used for restriction enzyme analysis were isolated and purified using ethidium bromide (EtBr) and phenol, as described by Zhang and Kerr (53). In all other cases, large- and small-scale isolations of plasmids from *Agrobacterium* strains

were performed as described by Hayman and Farrand (54). Plasmid preparations for DNA sequence analysis were further purified by two rounds of centrifugation in cesium chloride-EtBr. The EtBr was removed by extraction with equal volumes of isopropanol saturated with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the samples were dialyzed against LTE (10 mM Tris, 1 mM EDTA, pH 8.0).

Sequencing of plasmid DNA. The complete nucleotide sequence of pAoF64/95 was determined at the Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign (UIUC). Briefly, the purified plasmid DNA was sheared with a nebulizer, and the ends were repaired and dephosphorylated. The fragmented DNA was separated on an agarose gel, and fragments with sizes between 1.5 and 5.0 kb were selected for and cloned into pCR-Blunt II-TOPO (Invitrogen). Sequencing was done from both ends of the insert in vectors, using the Sanger dideoxy method. Sequences were assigned a quality score with Phred (55, 56), and clean sequences were assembled using PHRAP (57, 58). After final assembly, open reading frames (ORFs) were called using Glimmer (version 3.02 [59, 60; http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi]). We used BLAST to search the NCBI nonredundant protein sequence database. All other sequencing was done by the UIUC Core sequencing facility or by ACGT, Inc. Primers were ordered from Integrated DNA Technologies, Inc., or from the UIUC Core sequencing facility.

DNA manipulations. *Agrobacterium* strains were transformed using the freeze-thaw method (61), and *E. coli* was made competent using the calcium chloride method (52). In some cases, plasmids were mobilized from *E. coli* into *Agrobacterium* using a filter-mating technique (8). An overlapping cosmid bank of pAoF64/95 was constructed by cloning a Sau3AI partial digest of purified plasmid DNA into the cosmid vector pCP13b (Table 1) digested with BamHI and calf intestinal alkaline phosphatase (CIP). The clones were then packaged into λ using the Packagene λ DNA-packaging system (Promega) and transfected into *E. coli* strain LE392. Cosmid clones were mapped by restriction digestion, and the ends of appropriate inserts were sequenced using primers complementary to vector sequences.

Construction of mutant strains. All PCRs described here used *Pfu* polymerase (Stratagene). An in-frame deletion of *traR* was constructed on cosmid clone pMWS110 by the method of Datsenko and Wanner (63). The kanamycin cassette of pKD4 was amplified using the following primers, which contained 5′ overhang sequences of *traR*: forward primer, 5′ GTGGACGGTGACCTTCGCTCACTCATCGACATGACAGAAGTGTA GGCTGGAGCTGCTTCG 3′, and reverse primer, 5′ CTACAGCAGGCC GTGGTCCTTGGCGATCGCGACGAGGTGCATATGAATATCCTCCT TAGT 3′. The PCR product was transformed into *E. coli*(pKD46, pMWS110) following the published protocol (63), and alleles of *traR* in which the cassette was inserted into pMWS110 were selected for by resistance to kanamycin. Mutations were confirmed by restriction analysis and PCR amplification from the regions upstream and downstream of *traR* using the following primers: traRcheckup, 5′ AGCTCGCGAGGACTTG AATACCCGG 3′, and traRcheckdwn, 5′ GATCGCTGCGATCAGAGAG CACCG 3′. Cosmid clone pMWS110 contains *repABC* of pAoF64/95, which poses a plasmid incompatibility problem with pAoF64/95. Therefore, a 6.9-kb SpeI fragment containing the kanamycin-marked indel mutation of *traR* was subcloned into pWM91 (64). The resulting plasmid was transformed into *E. coli* S17-1/λpir, and the plasmid was transferred into NTL4(pAoF64/95) by filter mating (8). Transconjugants were selected on solid ABM medium containing kanamycin. Strains with double crossovers were screened for by their ability to grow on sucrose and by sensitivity to carbenicillin while retaining resistance to kanamycin. Mutants were confirmed by PCR amplification using the traRcheckup and traRcheckdwn primers. The mutant megaplasmid pAoF64/95Δ*traR* was genetically purified by isolation and subsequent transformation into strain NTL4.

The *traM* mutant was constructed using vectors described by Kalorgeraki and Winans (65). Briefly, an internal fragment of *traM* was amplified by PCR using primers that contained EcoRI and SalI sites (underlined): traMleft, 5′ GATCCAGAATTCGTCAGAACTGGAGGCTCTGG 3′ , and traMright, 5′ GATCCAGTCGACTACAGAACTCGACACCGCAG 3′ . The PCR fragment was directionally cloned into pVik107, resulting in an internal portion of *traM* fused in frame to *lacZ* on pVik107. This construct was transformed into *E. coli* strain S17-1/λpir, and the resulting strain was mated with NTL4(pAoF64/95) to yield NTL4(pAoF64/95::*traM*pVik107). The single-crossover mutation was confirmed by sequence analysis, and the mutated plasmid was genetically isolated by transformation into NTL4.

To complement the mutant strains, *traR* was amplified by PCR with traRpSRKGmsense (5′ GCCGGAATTCATATGGACGGTGACCTTC GCTC 3′) and traRpSRKGmantisense (5′ CGCGAATTCGGATCCTACAG CAGGCCGTGGTCCT 3′. The *traM* gene was separately amplified by PCR using the following primers: traMpSRKGmsense (5′ GCCCGAGCTCATAT GAGCGACGTGAACTCGTCTG 3′) and traMpSRKGmantisense (5′ GCG GAGCTCGGATCCTCAATCACCGACTTCGGGGGC 3′). The *traR* and *traM* genes were directionally cloned into pSRKGm (66) using NdeI and BamHI (the sites are underlined). In each case, the cloning results in fusion of the native start codon of the gene to the start codon of *lacZ*α in the vector. This arrangement puts expression of the cloned gene solely under the control of LacI. Cloning was confirmed by sequence analysis, and the constructs were transformed into the appropriate mutant strain. In complementation experiments, expression of the cloned gene was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM to the cultures.

Nucleotide sequence accession number. The full sequence of pAoF64/95 is available in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/) under accession number JX683454.

RESULTS

Identification and characterization of *Agrobacterium* isolates in which mannopine induces conjugation. We assessed a collection of mannopine-utilizing field isolates of *Agrobacterium* spp. for strains in which MOP induced conjugative transfer of the opine utilization trait. Of the isolates we tested, five transferred the trait to *A. tumefaciens* C58C1RS in an opine-inducible manner (Table 2). These strains were part of a collection of 11 isolates obtained mostly from apple and nut orchards in California and Oregon (the Corvallis strains). Two of the remaining six strains exhibited constitutive low-frequency transfer of MOP utilization, while four strains failed to transfer the trait under the culture conditions tested (Table 2).

All but 2 of the 11 Corvallis isolates were classified as biovar 2 agrobacteria. Six of the isolates induced tumors on at least one of three plant hosts tested (Table 2). Of the nine isolates tested, five were susceptible to agrocin 84, an antiagrobacterial antibiotic susceptibility to which is conferred by particular plasmid-encoded opine-catabolic systems (Table 2) (67, 68). Detailed growth studies of the five MOP-inducible isolates indicated that all of them also utilize MOA and AGA, but none utilize AGR (see Fig. S1A in the supplemental material). Consistent with this pattern, none of the five isolates expressed detectable levels of mannopine cyclase activity (see Fig. S1B in the supplemental material).

Mannopine catabolism and its inducible transfer are associated with a family of closely related plasmids. Gel electrophoretic analysis indicated that all five strains in which MOP induces conjugative transfer harbor at least one plasmid between 150 and 200 kb in size (Fig. 1A). Given the possibility that one or more of the isolates harbors more than one plasmid migrating in a single band, we genetically isolated the plasmids responsible for MOP catabolism by transforming strain NTL6, a plasmidless derivative of *A. tumefaciens* strain NTL4 (Table 1), with total plasmid DNA isolated from each strain, selecting directly for MOP utilization. MOP-utilizing

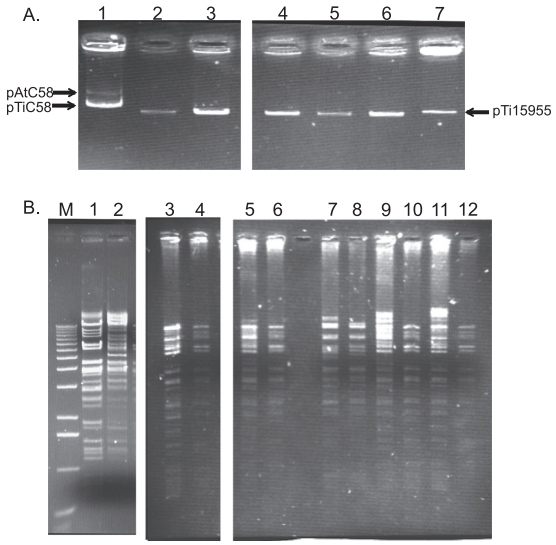

FIG 1 Isolates that transfer MOP catabolism harbor related plasmids. (A) Plasmid profiles of the MOP-inducible isolates. Plasmid DNA was isolated from the wild-type isolates. The lanes contain DNA from NTL4(pTiC58) (lane 1), F64/95 (lane 2), F265/93 (lane 3), J62/95 (lane 4), J84/95 (lane 5), M200/94 (lane 6), and 15955 (lane 7). The standards are pAtC58 (543 kb) and pTiC58 (214 kb) of strain C58 (lane 1) and pTi15955 (194 kb) of strain 15955 (lane 7). (B) BamHI fragment patterns of total plasmid DNA isolated from the wild-type isolates and the genetically isolated MOP catabolism plasmid from each wild-type strain. Total plasmid DNA was extracted from each wild-type isolate and an NTL6 transformant of the MOP catabolism plasmid from each strain and digested with BamHI, and the fragments were separated on a 0.8% agarose gel. Lanes: M, 1-kb ladder (Invitrogen); 1, NTL4(pAoF64/95); 2, C58C1RS(pArA4); 3, F64/95; 4, NTL6(pAoF64/95); 5, J84/95; 6, NTL6(pAoJ84/95); 7, F265/93; 8, NTL6(pAoF265/93); 9, J62/95; 10, NTL6(pAoJ62/95); 11, M200/94; 12, NTL6(pAoM200/94).

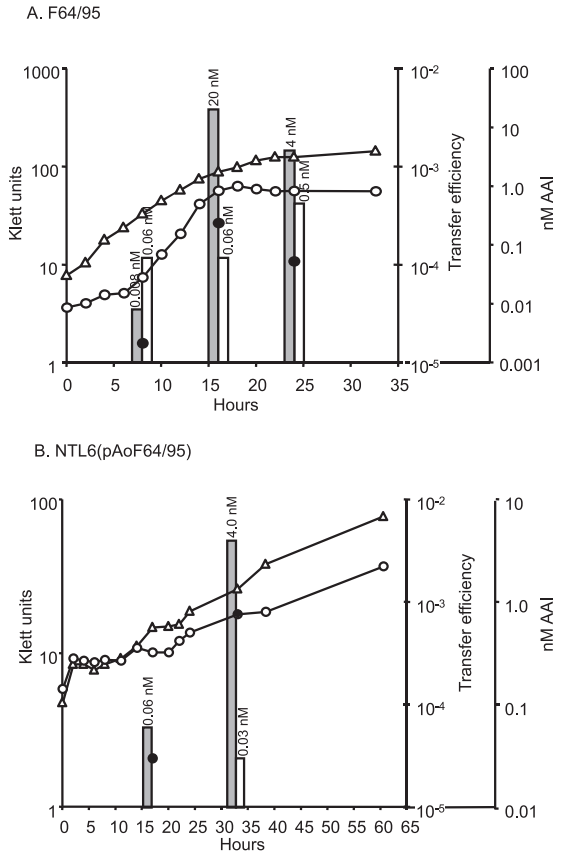

FIG 2 Growth with mannopine induces production of AAI and conjugative transfer of pAoF64/95. Strains F64/95 (A) and NTL6(pAoF64/95) (B) were grown in AB medium with 0.005% yeast extract and mannitol (open triangles and open bars) or MOP (open circles and gray bars). Growth was followed turbidometrically using a Klett colorimeter. At approximately 8-h intervals, a portion of each culture was removed and assayed for conjugative-transfer efficiency (black circles) and accumulation of AAI (bars). The amount of extractable AAI in the culture supernatant is indicated over the corresponding bar. The transfer frequency is expressed as the number of transconjugates recovered per input donor cell.

transformants were isolated in each case, and individual colonies from each transformation tested also utilized MOA and AGA, but not AGR (see Fig. S1A in the supplemental material).

To gauge the plasmid complement of the field isolates and the nature of the plasmids encoding opine utilization and MOP-inducible conjugation, we subjected plasmid samples from each of the five field isolates and one corresponding NTL6 transformant of each to restriction enzyme analysis. In all cases, the plasmids in the transformants yielded a fragment pattern that formed a subset of the pattern seen in the plasmid preparations from the corresponding field isolates (Fig. 1B, compare odd- and even-numbered lanes). Moreover, the fragment patterns of plasmids from the five different transformants were very similar to each other (Fig. 1B, odd-numbered lanes). These results strongly suggest that most, if not all, of the five field isolates tested harbor at least two plasmids of about the same size and that one of these plasmids, which is strongly conserved among the five isolates, is conjugative and encodes utilization of MOP, MOA, and AGA.

Given the apparent relatedness of the mannopine-catabolic plasmids, we focused on one wild-type field isolate, F64/95, and its MOP-catabolic plasmid, which we named pAoF64/95.

Growth with mannopine induces production of an acyl-homoserine lactone, as well as conjugative transfer of pAoF64/95. In all conjugative Ti and opine-catabolic plasmids studied to date, transfer is controlled by opines through regulation of an acyl-homoserine lactone-dependent quorum-sensing system (21, 23, 28, 29, 47). Preliminary studies indicated that growth of strain F64/95 with mannopine resulted in the production of an acyl-HSL that has the chromatographic properties of 3-oxo-C8-HSL (see Fig. S2 in the supplemental material). We assessed the conjugative-transfer characteristics of pAoF64/95 and whether opine-induced transfer was associated with induction of production of this acyl-HSL quormone. The field isolate and one NTL6 transformant harboring pAoF64/95 were cultured in minimal medium containing either mannitol or MOP as the primary source of carbon. At intervals, samples were removed and assayed for growth by viable-cell counts, and the cells were tested for conjugative competence. In addition, the culture supernatants were assayed quantitatively for acyl-HSLs, as described in Materials and Methods.

Strain NTL6 lacking pAoF64/95 grew well with mannitol but failed to grow with MOP (data not shown). In contrast, the field isolate and NTL6(pAoF64/95) grew almost as well with MOP as with mannitol (Fig. 2). Either plasmid-containing strain produced only barely detectable levels of 3-oxo-C8-HSL when grown with mannitol but accumulated steadily increasing amounts of the quormone as growth proceeded in medium containing MOP (Fig. 2). Concomitant with the increasing levels of the acyl-HSL signal, donors grown

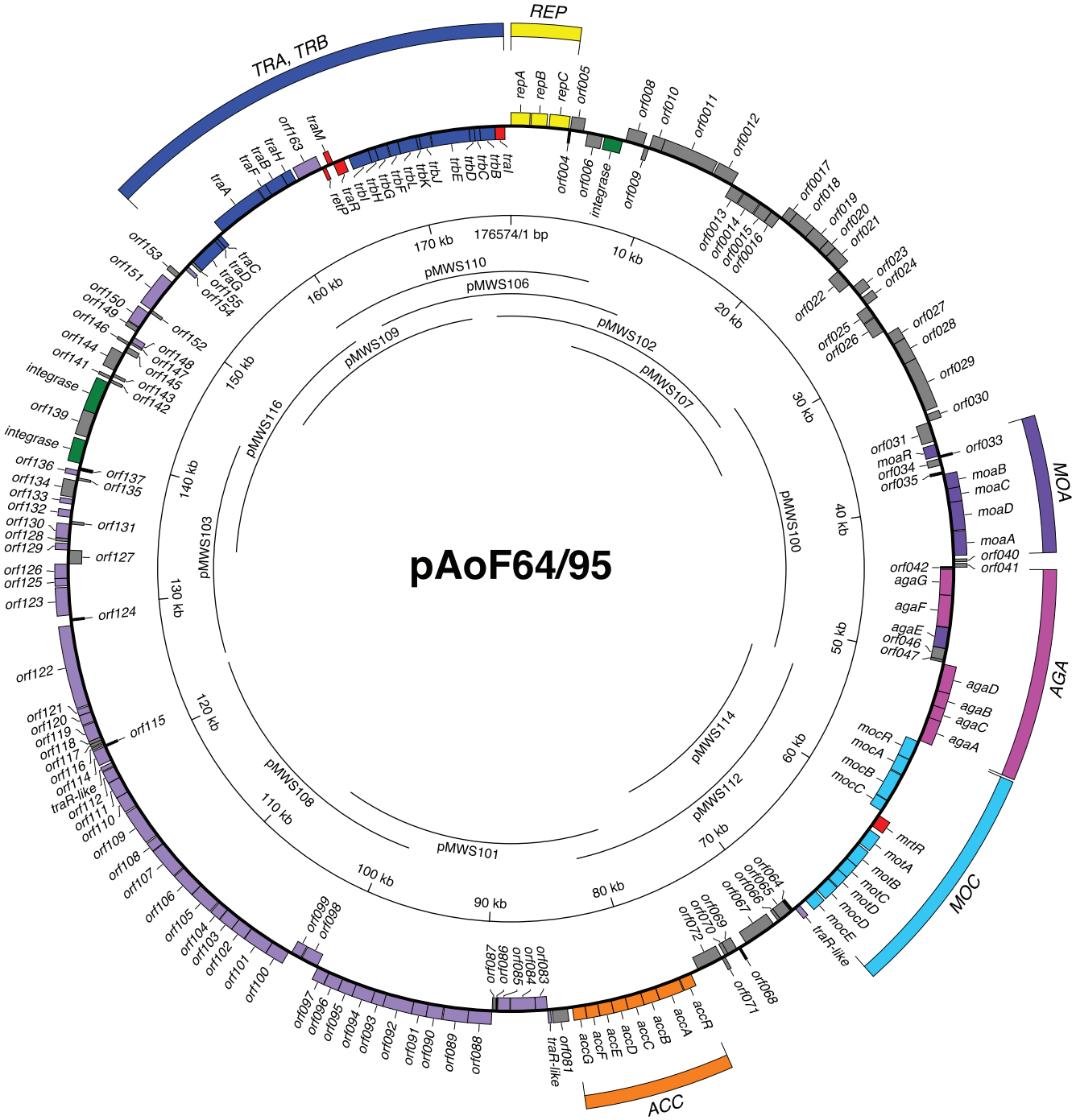

FIG 3 Physicogenetic map of pAoF64/95. The complete 176,574-bp sequence of pAoF64/95 was determined, and the sequence was annotated as described in Materials and Methods. Significant open reading frames are shown as boxes, with those on the outside oriented in a clockwise direction and those on the inside oriented in a counterclockwise direction. The genes are color coded with respect to known or putative functions as follows: yellow, replication; violet, uptake and catabolism of MOA; magenta, uptake and catabolism of AGA; cyan, uptake and catabolism of MOP; orange, uptake and catabolism of agrocinopines; blue, conjugative transfer; red, regulation of MOP catabolism or conjugative transfer; green, three putative integrase genes; lavender, a segment that is largely syntenic in gene composition and order with similarly sized regions of two Ri plasmids, pRi1724 and pRi2659; gray, other genes that have a returned hit in the NCBI nonredundant sequence database; black, ORFs with no significant similarities to genes in the databases. The extents of the inserts in each cosmid in the ordered library are represented by the arcs within the rings. The annotated sequence is available in GenBank (see the text).

with MOP transferred pAoF64/95 at increasing frequencies as growth continued (Fig. 2). Donors grown with mannitol failed to transfer the plasmid at a detectable level at any stage of growth tested.

Plasmid pAoF64/95 is an opine-catabolic element. We determined the nucleotide sequence of pAoF64/95. The plasmid, with a size of 176,574 bp, codes for 178 annotatable open reading frames (Fig. 3). We also constructed an overlapping cosmid clone bank of the plasmid (Fig. 3). Like most, if not all, large plasmids in the

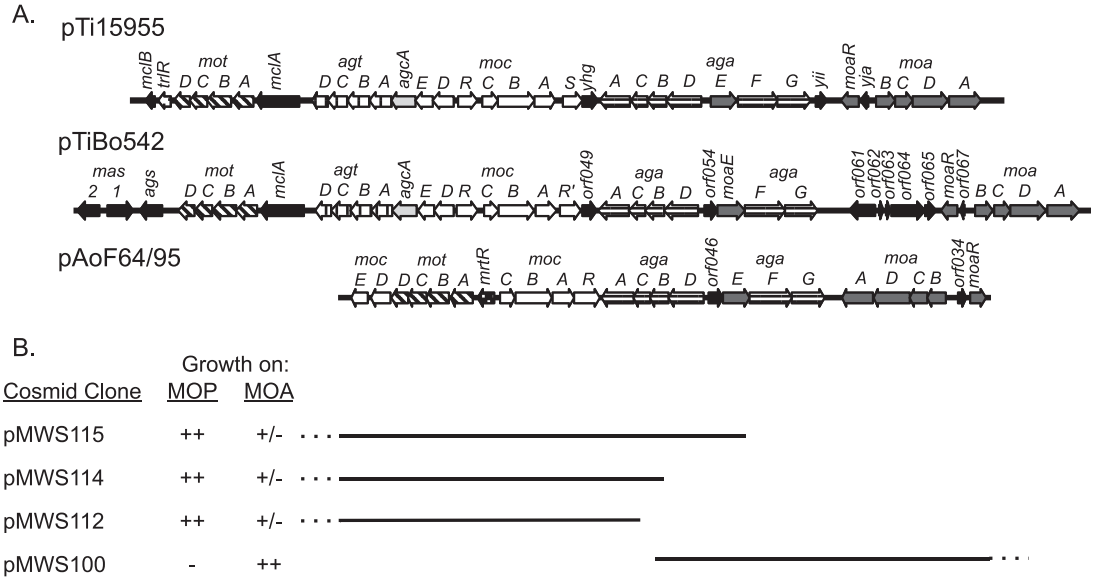

FIG 4 The mannityl opine catabolism region of pAoF64/95 is related to those of other *Agrobacterium* plasmids. (A) Alignment of the regions coding for the catabolism of the mannityl opines from two Ti plasmids, pTi15955 and pTiBo542, and from pAoF64/95. Orthologous genes and gene systems are depicted as arrows with identical fill patterns. Genes with common shading are involved in the same opine-catabolic pathways. *mot*, mannopine transport; *agt*, agropine transport; *acgA*, mannopine cyclase; *moc*, mannopine catabolism; *aga*, transport and catabolism of agropinic acid and mannopinic acid; *moa*, transport of mannopinic acid. All genes not directly involved in the catabolism and transport of the mannityl opines are in black. *mrtR*, a novel gene in pAoF64/95, codes for a member of the GntR family of regulators. *trlR* is a frame-shifted allele of *traR* in pTi15955 (30, 31). (B) A set of cosmids with overlapping inserts define the catabolic region of pAoF64/95. Derivatives of strain NTL4 harboring each cosmid were tested for the ability to grow on AB minimal agar containing either MOP or MOA as the sole carbon source. ++, growth as good as that of a known mannityl opine utilizer; +/−, very poor growth; −, no significant growth. The dotted lines indicate where the cosmid extends beyond the scope of the map.

family *Rhizobiaceae*, pAoF64/95 encodes a *repABC*-type replication system. Consistent with our observation that F64/95 fails to induce tumors, pAoF64/95 lacks a T region and all known components of the *vir* regulon.

Consistent with the observation that pAoF64/95 confers catabolism of three of the four mannityl opines, the plasmid contains a contiguous, approximately 30-kb region comprising 22 genes organized in six groups that is closely related to regions of Ti plasmids known to confer catabolism of MOP, MOA, and AGA (Fig. 3 and 4). The plasmid does not contain *agcA*, encoding catabolic mannopine cyclase (69–71), or the four-gene *agt* operon required for agropine uptake (51, 69), consistent with our observation that strains harboring pAoF64/95 do not catabolize the lactone opine. The organization of the two *aga* operons is virtually identical to those of pTi15955 and pTiBo542 (Fig. 4). However, the region comprising the *moa* transport genes and *moaR* is inverted in comparison to that of the two Ti plasmids. The 10 genes of pTi15955 comprising the MOP-catabolic regulon all are present in pAoF64/95. However, while they are grouped in similar units, their orientations and fine structures differ (Fig. 4). In pAoF64/95, the *mocDE* gene pair, which is organized as a two-gene operon in pTi15955, comprises the distal portion of the four-gene *mot* operon that encodes the MOP transporter. Notably, the *mocDE* gene pair replaces *trlR* at the end of the *mot* operon, and there is no other gene encoding a full-size TraR homolog in this region of the plasmid. Moreover, while the *moc* region of pTi15955 carries two closely related putative regulatory genes, *mocR* and *mocS* (72, 73), the corresponding region of pAoF64/95 carries only one such gene, which we call *mocR* (Fig. 4).

We confirmed that this region of pAoF64/95 is responsible for catabolism of the mannityl opines by analysis of the cosmid clone bank using two strategies. In the first, we electroporated an unordered pool of the cosmid bank into *A. tumefaciens* NTL4 and selected for progeny that could utilize MOP. Such transformants contained three cosmids, pMWS112, pMWS114, and pMWS115 (Fig. 3 and 4B). Restriction enzyme and sequence analyses showed that these three cosmids overlap the putative *moc* and *mot* genes and parts of the AGA transport and catabolism operons (Fig. 4B). In the second strategy, we transformed a cosmid, pMWS100, in which the insert overlapped the putative MOA catabolic region and parts of the AGA transport and catabolism operons, but not the MOP catabolism region (as depicted in Fig. 4B), into strain NTL4, selecting for resistance to tetracycline. We screened these transformants for the ability to utilize MOP and MOA. NTL4(pMWS100) utilized MOA but not MOP (Fig. 4B), which is consistent with our bioinformatics predictions for this cosmid clone.

Plasmid pAoF64/95 carries a second putative opine-catabolic region, *acc*, mapping between coordinates 77 and 84 kb (Fig. 3 and 5). On certain Ti and Ri plasmids, orthologous loci code for uptake and catabolism of the agrocinopines, a family of sugar phosphodiester opines (67, 74), as well as susceptibility to agrocin 84, the unique antiagrobacterial antibiotic (67, 68). Of the five strains with pAoF64/95-like plasmids, four were described to us by the Moore laboratory as being susceptible to agrocin 84, while one was not tested. In our assays, the field isolate F64/95 showed weak susceptibility to the antibiotic, only sometimes producing cloudy zones of growth inhibition (Fig. 5B). Strain NTL4(pAoF64/95) produced similar, although often more defined, zones of growth inhibition (Fig. 5B).

pAoF64/95 encodes a Ti plasmid-like conjugative-transfer system and all of the components of a TraR-TraI quorum-sensing regulatory system. By bioinformatics analysis, pAoF64/95 en-

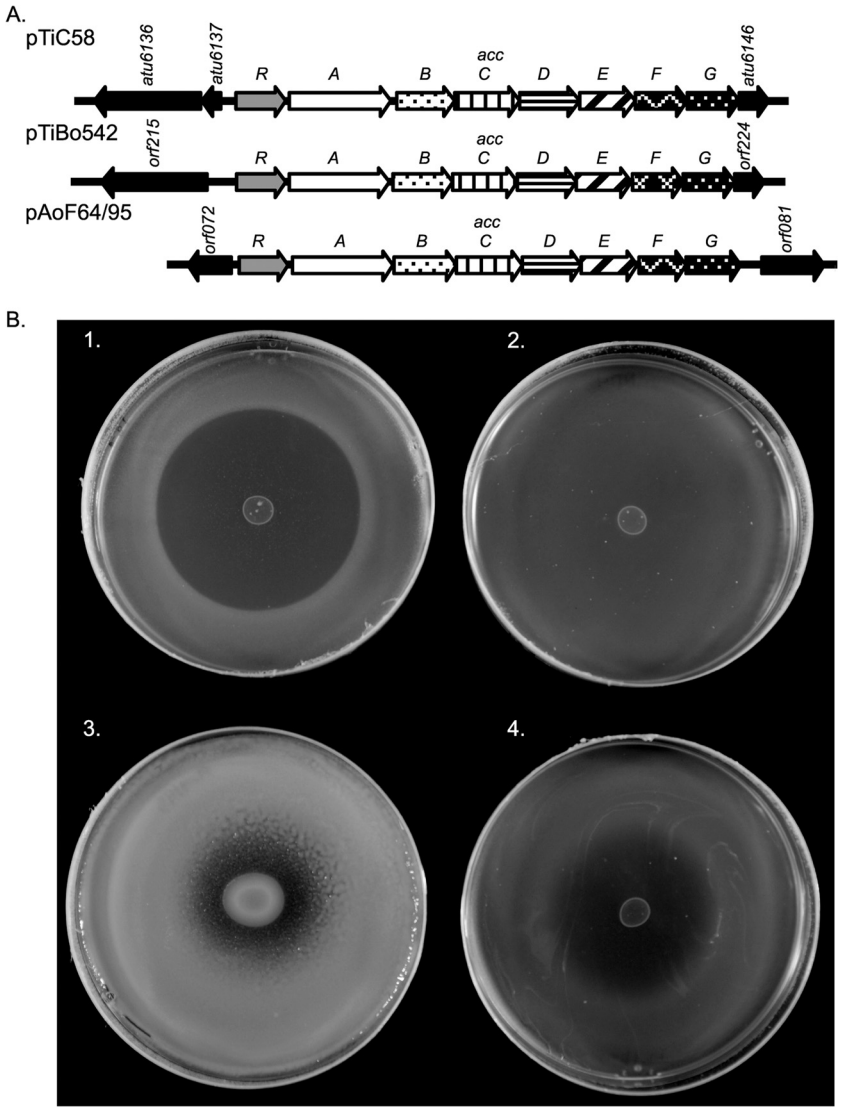

FIG 5 pAoF64/95 carries a locus related to the agrocinopine A and B catabolism operon. (A) Alignment of the *acc* operons from pTiC58, pTiBo542, and pAoF64/95. *accR* genes of pTiC58 and pTiBo542 encode the repressor that coregulates opine catabolism and conjugative transfer. Genes encoding orthologous functions are shown with the same fill pattern, while genes not directly involved in catabolism and transport are in black. (B) Agrocin 84 sensitivity assays. Strains were assessed for sensitivity to agrocin 84 as described in Materials and Methods. 1, C58; 2, NTL4; 3, F64/95; 4, NTL4(pAoF64/95).

codes a single identifiable conjugative-transfer system, and this system is virtually identical in gene content and operon organization to those of other Ti, Ri, and opine-catabolic plasmids (Fig. 6). The 12-gene *trb* operon, encoding an IncP-like type IV secretion system, is adjacent to and oriented divergently from the *repA* gene of the replication operon (Fig. 3 and 6). The two DNA-processing operons, *traAFBH* and *traCDG*, are adjacent to and divergently oriented with respect to each other, although the latter carries an additional putative small open reading frame, *orf155*, not present in the *traCDG* operons of other Ti plasmid-like systems (Fig. 3 and 6). The *traA* and *traC* genes are separated by a 254-bp intergenic region that contains a sequence similar to that of the *oriT* regions of pTiC58 and pTiR10 (75, 76) (Fig. 7).

The conjugative-transfer region also carries the three genes *traR*, *traI*, and *traM*, which in studied Ti, opine-catabolic, and some *Rhizobium* plasmids are responsible for regulating conjugative transfer in a quorum-dependent manner (Fig. 6). Moreover, the general locations and orientations of the three genes in pAoF64/95 closely resemble those in other known conjugative elements. *traI*, which encodes the acyl-HSL synthase (24), is the first gene of the *trb* operon, and *traR* and *traM*, encoding the activator and antiactivator, are closely linked, convergently oriented, and located just distal to the *traAFBH* operon. However, unlike the gene organization in systems in which conjugative transfer is opine inducible, *traR* appears to be monocistronic and is certainly not a member of a plasmid-specific opine-regulated operon (Fig. 6).

We assessed the activity of *traI* by examining a set of overlapping cosmid clones that comprise the *tra*, *trb*, *traI*, and *repABC* regions of pAoF64/95 for production of the acyl-HSL quormone. Derivatives of strain NTL4 harboring cosmids pMWS106 and pMWS110, both of which carry *traI*, produced low but detectable levels of the signal, while the strain harboring pMWS109, which maps to the same region but does not

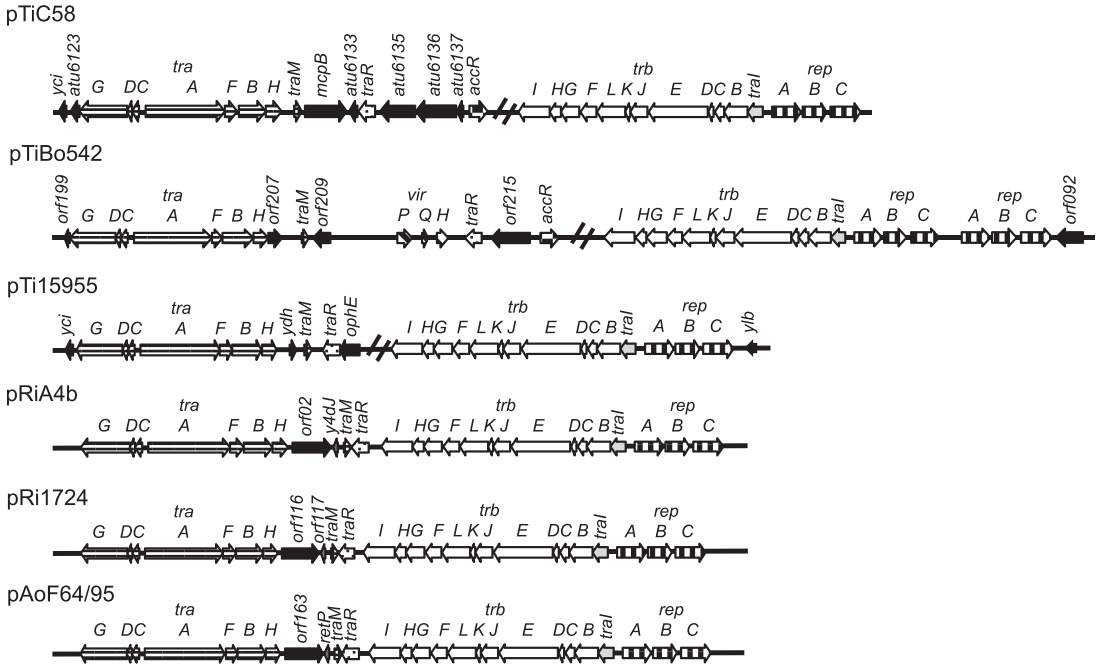

FIG 6 The *tra* and *trb* operons of Ti, Ri, and opine-catabolic plasmids are conserved. The regions coding for DNA metabolism (*tra*) and mating-pair formation (*trb*) from three Ti plasmids (pTiC58, pTiBo542, and pTi15955), two Ri plasmids (pRiA4b and pRi1724), and pAoF64/95 were aligned. Orthologous genes and gene systems are depicted as arrows with identical fill patterns. The genes known to be involved in regulation of Ti plasmid transfer are *traM*, *traR*, and *traI*. The plasmid replication genes are denoted *rep*. In pTiC58 and pTiBo542, the product of *accR* represses the *acc* and *arc* operons (13). The two Ri plasmids and pAoF64/95 carry a small helix-turn-helix *xre* family gene labeled *y4dJ*, *orf117*, and *retP*. Novel genes not yet characterized are filled in black. The double slash lines on the Ti plasmids indicate where the *tra* and *trb* regions are separated on those plasmids.

overlap *traI*, failed to produce the acyl-HSL (see Fig. S3 in the supplemental material).

The *traR* gene product is required for the MOP-dependent induction of AAI production and conjugative transfer. In the known Ti plasmid conjugative-transfer systems, TraR directly activates transcription of the *traItrb* operon and the two divergently oriented *tra* operons (15–20). We constructed a *traR* mutant by creating an in-frame, kanamycin-marked deletion derivative of the gene on the megaplasmid. Donors harboring this construct failed to transfer the plasmid at detectable frequencies, even when grown with MOP (Fig. 8A). Moreover, these donors failed to produce elevated levels of the acyl-HSL signal under any growth conditions tested (Fig. 8B). These results suggest that a functional TraR is required to express *traI* and the *tra* and *trb* genes.

The *traR* mutation was fully complementable with the wild-type allele cloned into pSRKGm. When this merodiploid construct was pregrown with MOP as the primary carbon source and expression of the recombinant *traR* was induced with IPTG, conjugative transfer and acyl-HSL production were restored to approximately wild-type frequencies (Fig. 8C and D). Surprisingly, when the strain was grown on mannitol and *traR* was induced with IPTG, conjugative transfer and induction of AAI production remained undetectable (Fig. 8C and D).

FIG 7 The *oriT* region of pAoF64/95 is strongly conserved with those of two Ti plasmids, pTiC58 (75) and pTiR10 (76). Nucleotides identical in all three regions are shaded black. Nucleotides conserved in two of the three regions are shaded gray. The *nic* site of pTiR10 (76) is indicated by an arrowhead.

TraM negatively regulates conjugative transfer of pAoF64/95. In the known conjugative-transfer systems of *Agrobacterium*, TraM inhibits the activity of TraR by directly binding to the activator (27, 77). We created a disruption mutant of *traM* on pAoF64/95 [strain NTL4(pAoF64/95::*traM*pVik107)] as described in Materials and Methods and assayed the mutant for AAI production and conjugative transfer following growth with MOP or with mannitol as the primary carbon source. Consistent with its role as an antiactivator, the *traM* mutant produced high levels of AAI and transferred the plasmid at high frequency, even when grown with mannitol, a noninducing substrate (Fig. 9A and B).

In initial studies, an IPTG-inducible copy of *traM* cloned into pSRKGm did not fully complement the *traM* mutant (data not shown). We reasoned that since the mutant is constitutive for transfer, to abolish transfer, *traM* would have to be expressed over many generations to dilute out the donors that had already produced fully active conjugative-transfer systems (78). To test this, we passaged the complemented mutant in AB minimal medium with either mannitol or mannopine through sequential subcultures, either continually with or without IPTG. We tested samples for conjugative competence before each dilution step. When no IPTG was added, transfer and AAI levels remained high regardless of whether the cells were grown with mannitol or MOP (Fig. 9C and D). Donor cells grown with IPTG continued to transfer the plasmid, though at lower frequency, over the first 10 to 20 generations. However, after passage of the strain through approximately 20 to 30 generations with inducing levels of IPTG, conjugative transfer was reduced to undetectable levels, even when the

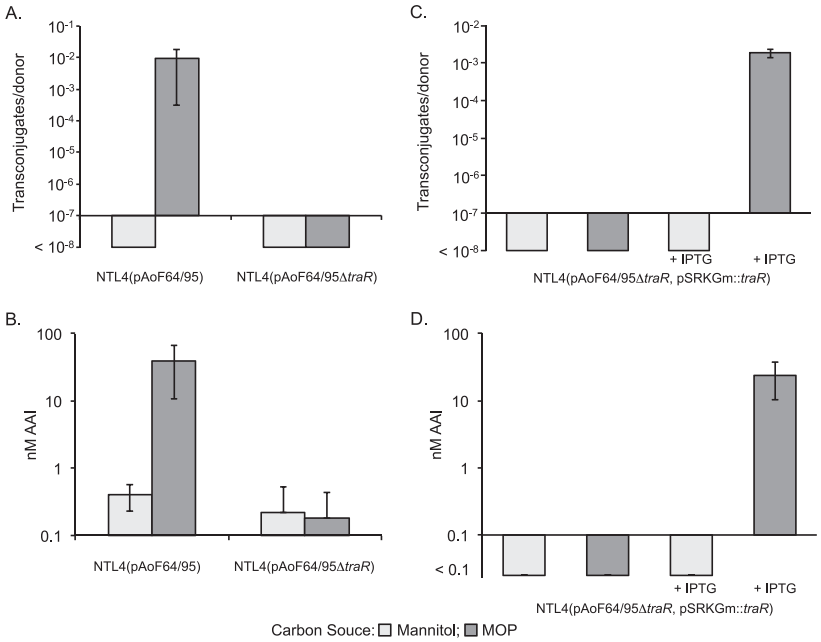

FIG 8 TraR is essential for induction of acyl-HSL production and conjugative transfer. (A and B) NTL4(pAoF64/95) and the *traR* mutant, NTL4(pAoF64/95Δ*traR*), were grown in AB medium containing mannitol or MOP as the primary carbon source. After overnight growth, samples were removed and tested for conjugative transfer using *A. tumefaciens* strain C58C1RS as the recipient (A) and levels of AAI accumulation (B). (C and D) The *traR* mutant was complemented with a wild-type copy of *traR* cloned into pSRKGm. The resulting strain, NTL4(pAoF64/95Δ*traR*, pSRKGm::*traR*), was grown in AB minimal medium containing mannitol or MOP, each with and without IPTG, and assessed for conjugative transfer (C) and AAI accumulation (D). The experiment was done two or three times, and the mean and standard deviation for each culture condition are shown.

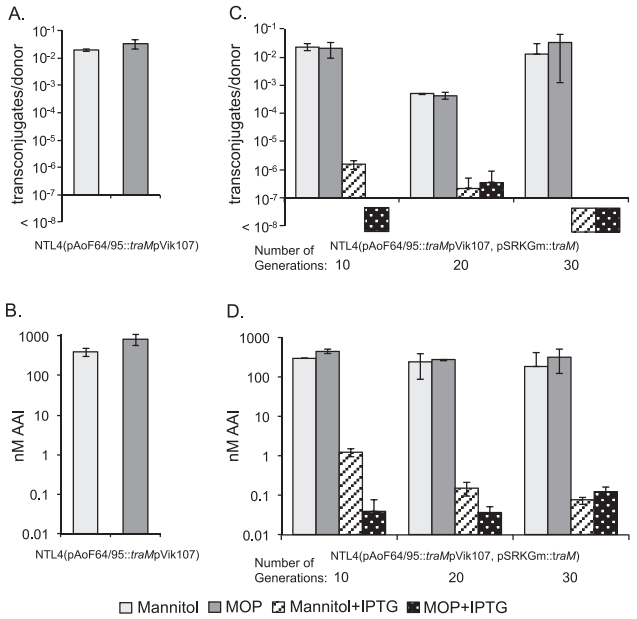

FIG 9 TraM inhibits TraR-dependent induction of AAI production and conjugative transfer. (A and B) The *traM* mutant, NTL4(pAoF64/95::*traM*pVik107), was assessed for conjugative transfer (A) and AAI accumulation (B) when grown on AB medium supplemented with either mannitol or MOP. (C and D) The mutant was complemented in *trans* with a wild-type copy of *traM* cloned into pSRKGm. This strain, NTL4(pAoF64/95::*traM*pVik107, pSRKGm::*traM*), was continuously cultured in AB minimal medium supplemented with either mannitol or MOP and with or without IPTG to induce expression of *traM*, as described in Materials and Methods. Samples were removed after the indicated numbers of cumulative generations, and the cells were tested for conjugative competence (C) and AAI accumulation (D), as described in Materials and Methods. The numbers of generations are approximate. Each experiment was done two or three times, and the means and standard deviations are shown.

cells were grown with MOP (Fig. 9C and D). We reasoned that overexpressing TraM should keep conjugative transfer repressed, even in cells grown with the inducing opine (Fig. 9C).

DISCUSSION

Five independently isolated opine-catabolic plasmids confer MOP utilization and are highly similar to each other. The existence of *trlR*, a mutant allele of *traR* associated with the *mot* operon of octopine-type Ti plasmids, led us to the hypothesis that *Agrobacterium* plasmids in which quorum-dependent conjugative transfer is induced by MOP would exist. The prediction proved accurate; of 11 wild isolates of mannopine-utilizing *Agrobacterium* spp. obtained from Oregon State University, five conjugatively transferred the catabolic trait only when grown with MOP (Table 2). In all five cases, the transmissible trait is associated with a large conjugative plasmid, and as judged by restriction enzyme analysis (Fig. 1), these five MOP-inducible plasmids are closely related. All five of the isolates are biovar 2 strains, but only two are demonstrably pathogenic (Table 2). Of the remaining six isolates examined, four failed to transfer MOP utilization at a detectable frequency under the culture conditions tested, and two transferred the trait at a low opine-independent constitutive level (Table 2).

pAoF64/95 is an opine-catabolic plasmid that carries a conserved set of genes for transport and catabolism of the mannityl opines. Consistent with the phenotype, sequence analysis of one such opine-inducible plasmid, pAoF64/95, indicated that these elements are not Ti plasmids; pAoF64/95 lacks a T region and the genes of the *vir* regulon (Fig. 3). Instead, it is an opine-catabolic plasmid and carries genes for the uptake and catabolism of at least two families of these tumor-specific substrates, the agrocinopines and three of the four mannityl opines. Both loci confer the expected phenotypes; the

acc operon confers sensitivity to agrocin 84, a marker for catabolism of the agrocinopine opines (67) (Fig. 5), while the *moc* locus confers utilization of MOP, MOA, and AGA (Fig. 4; see Fig. S1 in the supplemental material). Strain F64/95 does not utilize agropine, the fourth member of the mannityl opine family (see Fig. S1 in the supplemental material). Consistent with this observation, pAoF64/95 lacks *agcA*, which codes for the enzyme that converts agropine to MOP (69–71), and also the *agt* operon that encodes the agropine transporter (51, 69). While the overall organization of the *moc* locus is similar to that of the octopine-type Ti plasmids, there are some rearrangements (Fig. 4). Most significantly, the *mocDE* genes in pAoF64/95 are coupled with the 3′ end of the *mot* operon, and instead of two *mocR*-like genes, there is only one (Fig. 4). pAoF64/95 carries an additional novel gene in the mannopine catabolism region, *mrtR*, which codes for a product that aligns with the GntR family of transcriptional regulators. Perhaps MrtR contributes to the regulation of mannopine catabolism, MOP-inducible transfer, or both.

Given that growth with MOP induces transfer, we expected that, like *trlR*, *traR* of pAoF64/95 would be linked to the *mot* operon. However, on pAoF64/95, *traR* is monocistronic, is located between the contiguous *tra* and *trb* regions, and is approximately 66 kb removed from the closest gene in the *moc* operon (Fig. 3 and 6). The absence of a functional *traR* linked to a MOP-associated operon on pAoF64/95 does not preclude the existence of a plasmid with a gene organization similar to that of the *mot-ABCDtrlR* operon of octopine-type Ti plasmids. However, there currently is no evidence of such a *mot*-associated functional form of the *traR* gene on an *Agrobacterium* megaplasmid.

pAoF64/95 encodes a conserved conjugative-transfer system that is most similar in gene organization to Ri and *Rhizobium* plasmids. The pAoF64/95 plasmid additionally shares a set of core genes that are strongly conserved in a large group of plasmids found in *Agrobacterium* and *Rhizobium* isolates. These genes code for replication and conjugative transfer, as well as components associated with the quorum-dependent regulation of these functions. In a typical Ti plasmid, the two *tra* operons, *oriT*, *traR*, and *traM*, are located near a conjugative opine catabolism region of the plasmid. In these plasmids, *traR* is, without exception, located in an opine-inducible operon (28; reviewed in reference 7). The *trb* operon, which codes for the type IV mating bridge, as well as TraI, the acyl-HSL synthase, is invariably directly linked and oriented divergently from *repA* of the *repABC* operon (reviewed in reference 7). Moreover, in Ti plasmids, the *tra* complex and the *trb-rep* locus are separated by 60 to 85 kb of sequence.

The organization of these loci in pAoF64/95 more closely resembles that of megaplasmids from *Agrobacterium rhizogenes* isolates, including pRi1724, pRiA4 (Fig. 7), and pRi2659, as well as some *Rhizobium* plasmids, including pRL1JI (79) and p42a (80). In these plasmids, the *trb-repABC* complex and the two divergently oriented *tra* operons all are located in close association, with *traM* and a monocistronic *traR* located between the two conjugative-transfer regions (Fig. 6).

TraR is an activator but may be regulated differently from TraR in the Ti plasmid systems. Like the identified conjugative-transfer systems of the Ti plasmids, transfer of pAoF64/95 is regulated by a TraR-dependent mechanism. Mutational analysis clearly indicates that, as in Ti plasmid systems, *traR* is required for opine-mediated induction of conjugative transfer (Fig. 8). However, complementation analysis showed some differences from opine-mediated regulation of *traR* in Ti plasmids. When their cognate *traR* genes are overexpressed in strains harboring Ti plasmids, the opine signals are no longer required to induce transfer (23, 29, 47). Intriguingly, donors containing pAoF64/95Δ*traR* were complemented in *trans* with a cloned, fully induced copy of wild-type *traR*, but only when grown with MOP (Fig. 8). This continued requirement for the conjugative opine suggests that the opine-dependent regulatory system that controls TraR-mediated activation of conjugative transfer is novel and may require an additional MOP-dependent element.

Activation of transfer of pAoF64/95 is further modulated by the antiactivator, TraM. In strains harboring the archetypical Ti plasmids pTiC58 and pTiR10, the product of the *traM* gene modulates TraR activity. TraM functions as an antiactivator by direct interaction with TraR (27, 77); mutants lacking *traM* transfer constitutively, but at frequencies lower than those observed in wild-type strains induced by growth with their conjugative opine (25, 26). Apparently, TraR expressed at its basal level is sufficient to induce transfer, albeit at a low frequency. One role of TraM, then, is to sequester this low level of TraR, thereby preventing plasmid transfer in the absence of the conjugative opines (25, 26).

pAoF64/95 also carries a *traM* gene, and a *traM* mutant of the plasmid is constitutive for transfer (Fig. 9). When this mutant is complemented by overexpression of *traM*, transfer is abolished, even when the cells are grown with mannitol (Fig. 9). These results support a role for TraM as an antiactivator of TraR. In contrast to the Ti plasmid system, the *traM* mutant of pAoF64/95 exhibits constitutive transfer at higher than anticipated frequencies (Fig. 9). This observation, coupled with the data concerning complementation of the *traR* mutant (Fig. 8) discussed above, suggests that TraR is expressed at a basal level higher than that of the Ti plasmid systems and that perhaps the ratio of TraM to TraR is higher in pAoF64/95 than it is in strains harboring the Ti plasmids.

The pAoF64/95 group of plasmids and pArA4 of *A. rhizogenes* A4 are related. Based on our restriction enzyme analysis, pAoF64/95 defines a family of closely related opine-catabolic plasmids that are widely distributed among the agrobacteria. In addition to their presence in the subset of the Corvallis isolates in which MOP induces transfer, our results suggest that the members of this group are distributed among the classical mannityl-opine-utilizing strains of *A. rhizogenes*. Wild-type strain A4, isolated from the hairy roots of naturally infected rose plants (81) in California (82), has three well-studied plasmids: pRiA4, pArA4, and a cointegrate of the two (83). Based on restriction enzyme cleavage patterns, pArA4 is similar, but not identical, to pAoF64/95 and the other four MOP-inducible plasmids in this family (Fig. 2). Consistent with this physical relatedness, like pAoF64/95, the plasmid codes for catabolism of MOP, MOA, and AGA, but not agropine (48), as well as utilization of agrocinopines (54).

These related plasmids are all found in independent isolates of *Agrobacterium* spp. While the plasmids are not virulence elements, based on the pathogenicity properties of the host isolates (Table 2), they are present in strains that harbor Ri plasmids, Ti plasmids, and possibly other opine-catabolic plasmids. In strain A4, whose two plasmids have been studied in detail, the T-right region of the Ri plasmid carries the genes for synthesis by the plant neoplasia of all four mannityl opines and the agrocinopines (84, 85). pRiA4, however, confers utilization only of AGR, while pArA4 codes for the uptake and catabolism of AGA, MOA, MOP, and the agrocininopines (48). Thus, the catabolic properties of

pArA4 expand the range of the mannityl opines utilizable by strains harboring pRiA4. Additionally, opine-catabolic plasmids can provide an advantage to avirulent *Agrobacterium* strains, such as F64/95, by enabling the bacteria to utilize opines produced by neoplasias induced by a virulent strain of *Agrobacterium*. Such opine-utilizing, nonpathogenic isolates of *Agrobacterium* have been repeatedly cultured from pathogen-induced neoplasias (38, 86–89). The fact that these opine-catabolic plasmids responsible for the cheater phenotype of these strains so closely resemble Ti and Ri plasmids of the pathogen illustrates the genetic plasticity of the core replicon structure of these rhizobial elements.

A large region of pAoF64/95 is syntenic with a region from two Ri plasmids, indicating that pAoF64/95 is chimeric and that this region may encode advantageous functions. Including the core replication and transfer regions, a large portion of pAoF64/95 is highly syntenic with regions of two Ri plasmids, pRi1724 and pRi2659 (Fig. 3, lavender-colored genes). This region, about 94 kb in size, includes ORFs that are annotated as putative unknown opine transport and metabolic genes (90, 91). Likewise, the region contains sequences that align with sugar transporters and glycerol metabolism genes (90, 91), as well as a large number of hypothetical genes or genes of unknown function.

An 8.8-kb region located in the middle of this syntenic region is unique to pAoF64/95. This segment consists mainly of ORFs coding for hypothetical proteins and two putative phage integrase proteins (Fig. 3, genes in green around 140 kb). The presence of such genes in pAoF64/95 suggests that this small region was acquired by an insertion event.

All told, fully one half of pAoF64/95 is synonymous with syntenic regions of two Ri plasmids. The conservation of this large region is particularly interesting considering the varied nature of the plasmids and the geographical locations from which the parental strains were isolated. MAFF 301724, a biovar 1 strain from which pRi1724 was identified, was isolated in Japan from a melon plant with hairy root disease (92, 93). The parental strain of pRi2659, also a biovar 1 strain, was isolated in the United Kingdom from a similarly diseased cucumber plant (91). Based on sequence analysis, pRi1724 and pRi2659 are closely related plasmids (90). Strain F64/95, on the other hand, is a nonpathogenic biovar 2 isolate that was cultured from an apple crown gall in California (Table 2). The varied locations and strain backgrounds of the three isolates and the fact that this region is located on Ri and opine-catabolic plasmids suggest that the genes located on this segment confer some unknown but advantageous functions in the habitats occupied by these bacteria. Additionally, the fact that this region is conserved in three different plasmids with at least two different gene contexts supports the notion that the large megaplasmids in both *Rhizobium* and *Agrobacterium* are chimeric and evolved by recombination with other rhizobial plasmids (90, 94–97).

The mechanism of opine-inducible transfer of pAoF64/95 may be novel. In Ti plasmid systems, *traR* is transcriptionally controlled by opines through the simple fact that it is a member of an operon that is regulated by the opine-responsive regulatory element. However, in pAoF64/95, *traR* is monocistronic and is not located near the mannopine catabolism region. In addition, our mutational and complementation analyses of *traR* and *traM* suggest that *traR* expression may not be controlled directly at the transcriptional level by a MOP-responsive regulatory element. It is possible, for example, that MOP controls the expression of some other, still unidentified component of the regulatory circuitry.

This accumulating evidence supports a novel mode of regulation of transfer of pAoF64/95, and perhaps other plasmids of *Agrobacterium* spp. with transfer and regulatory genes organized in a similar fashion. A more detailed understanding of the regulation of the quorum-sensing system of pAoF64/95 may be useful, for example, in understanding the regulation of conjugative transfer among the Ri group of plasmids, which have all of the components of other conjugative plasmids.

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