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

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

Margaret E. Wetzela, Kun-Soo Kim,b,* Marilyn Miller,b Gary J. Olsen,a,c Stephen K. Farranda

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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, trIR, 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) quorumone. 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 conjugal 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 quorumone 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.

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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*<sub>noc</sub>, is in an operon induced by nopaline, while the other, *traR*<sub>arc</sub>, 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*<sub>noc</sub> mutant, conjugative transfer is still inducible by agrocinopines. Likewise, if *traR*<sub>arc</sub> 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*<sub>arc</sub>, is a member of an operon that is activated by octopine, and this opine induces *tra*<sub>R</sub><sup>B</sup><sub>arc</sub> product can dimerize with the full-size, functional TraR, 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*<sub>arc</sub>, is a member of an operon that is activated by octopine, and this opine induces *tra*<sub>R</sub><sup>B</sup> 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*<sub>noc</sub> mutant, conjugative transfer is still inducible by agrocinopines. Likewise, if *traR*<sub>arc</sub> is mutated, conjugative transfer remains inducible by nopaline, but not by the agrocinopines (28).

Based on the presence of *tri*<sub>R</sub> 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 (Fisher 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.

**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, production of AAI; Ampr, ampicillin resistance; Gmr, gentamicin resistance; Kan<sup>r</sup>, kanamycin resistance; Rif<sup>r</sup>, rifampin resistance; Sm<sup>r</sup>, streptomycin resistance; Tcr, tetracycline resistance; Ty<sup>p</sup>, trimethoprim resistance.

### TABLE 1 Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><em>Agrobacterium</em> sp.</td>
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<td></td>
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<tr>
<td>AFI/95</td>
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<td>L. Moore</td>
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<tr>
<td>AR11N/71</td>
<td>Wild-type mannopine-utilizing strain</td>
<td>L. Moore</td>
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<tr>
<td>B24/93</td>
<td>Wild-type mannopine-utilizing strain</td>
<td>L. Moore</td>
</tr>
<tr>
<td>B26/94</td>
<td>Wild-type mannopine-utilizing strain</td>
<td>L. Moore</td>
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<tr>
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<td>Wild-type mannopine-utilizing strain</td>
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<td>Wild-type mannopine-utilizing strain</td>
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<tr>
<td>J84/95</td>
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<td>M200/94</td>
<td>Wild-type mannopine-utilizing strain</td>
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<td>15955</td>
<td>Wild-type octopine strain; MOP&lt;sup&gt;B&lt;/sup&gt; MOA&lt;sup&gt;B&lt;/sup&gt; AGR&lt;sup&gt;B&lt;/sup&gt; AGA&lt;sup&gt;B&lt;/sup&gt;</td>
<td>OC</td>
</tr>
<tr>
<td>C58</td>
<td>Wild-type nopaline strain; pAtC58 pTiC58</td>
<td>OC</td>
</tr>
<tr>
<td>C58C1RS</td>
<td>Ti plasmidless derivative of C58; pAtC58</td>
<td>OC</td>
</tr>
<tr>
<td>Sm&lt;sup&gt;r&lt;/sup&gt; Rif&lt;sup&gt;r&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
<td>NTL4</td>
<td>Ti plasmidless derivative of C58; pAtC58 ΔtetAR</td>
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</tr>
<tr>
<td>NTL6</td>
<td>Plasmidless derivative of NTL4</td>
<td>OC</td>
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<tr>
<td><em>E. coli</em></td>
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<td>DH5α</td>
<td>Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(k&lt;sup&gt;R&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt;) supE44 thi-1 gyrA relA1 hsdR514 (r&lt;sup&gt;K&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt;) glvV (supE44) tryT (supF8) lacY1 or Δ(lacZΔM15 SsrB) galK2 galT22 metB1 thrB5</td>
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<td>S17-1 λpir</td>
<td>T&lt;sup&gt;φ&lt;/sup&gt;, Sm&lt;sup&gt;r&lt;/sup&gt; recA thi pro hsdR M&lt;sup&gt;+&lt;/sup&gt; RP4::2-Tc::Mu::Km Tn7 λpir</td>
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<td>J. Tempé</td>
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<td>Amp&lt;sup&gt;r&lt;/sup&gt;; A Red helper plasmid</td>
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<tr>
<td>pSRKGM</td>
<td>Gm&lt;sup&gt;r&lt;/sup&gt;; IPTG-inducible expression vector</td>
<td>66</td>
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<tr>
<td>pVJK107</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;; promoterless lacZY; pir dependent</td>
<td>65</td>
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<tr>
<td>pWM91</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;; sucB</td>
<td>64</td>
</tr>
<tr>
<td>pZL84</td>
<td>Gm&lt;sup&gt;r&lt;/sup&gt;; indicator for detection of acyl-HSL</td>
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*Tra<sup>r</sup>* production of AAI; Amp<sup>r</sup>, ampicillin resistance; Gm<sup>r</sup>, gentamicin resistance; Kan<sup>r</sup>, kanamycin resistance; Rif<sup>r</sup>, rifampin resistance; Sm<sup>r</sup>, streptomycin resistance; Tc<sup>r</sup>, tetracycline resistance; Ty<sup>p</sup>, trimethoprim resistance.

OC, our collection.
Monitoring growth. Strains F64/95 and NTL6(pAoF64/95) were pre-cultured 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 10⁷ 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 C₁₈ reversed-phase thin-layer chromatography (TLC) plate and separated using methanol-H₂O (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 NTL6(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).

Agrobacterium tumefaciens strain 15955 was minced and placed in 50-ml sterile conical tubes. Enough MilliQ 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 agroproline 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

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**TABLE 2 Characteristics of MOP-utilizing wild-type isolates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Opine(s) utilized</th>
<th>Conjugation frequency&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Isolation</th>
<th>Host</th>
<th>Location</th>
<th>Tumorogenic</th>
<th>Agrocin sensitivity</th>
<th>Biovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF1/95</td>
<td>MOP, OCT</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
<td>Lilac</td>
<td>Visalia, CA</td>
<td>Yes</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>AR11N7/1</td>
<td>MOP, NOP, OCT</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
<td>Apple</td>
<td>Canby, OR</td>
<td>Yes</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
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<tr>
<td>B24/93</td>
<td>MOP</td>
<td>3.3 x 10⁻⁶</td>
<td>Induced</td>
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<td>Portland, OR</td>
<td>No</td>
<td>No</td>
<td>2</td>
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<tr>
<td>B26/94</td>
<td>MOP, NOP</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
<td>Quince</td>
<td>Portland, OR</td>
<td>Yes</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>B98/94</td>
<td>MOP</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
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<td>Gridley, CA</td>
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<td>No</td>
<td>2</td>
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<tr>
<td>B155/93</td>
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<td>Uninduced</td>
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<td>Portland, OR</td>
<td>No</td>
<td>Yes</td>
<td>ND</td>
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<tr>
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<td>Modesto, CA</td>
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<td>Yes</td>
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<tr>
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<td>Uninduced</td>
<td>Apple</td>
<td>Yamhill, OR</td>
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<td>Yes</td>
<td>2</td>
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<tr>
<td>J62/95</td>
<td>MOP, NOP</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
<td>Apple</td>
<td>Modesto, CA</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
<tr>
<td>J84/95</td>
<td>MOP, NOP</td>
<td>&lt;10⁻⁸</td>
<td>Uninduced</td>
<td>Apple</td>
<td>Woodburn, OR</td>
<td>No</td>
<td>ND</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> NOP, nopaline; OCT, octopine.

<sup>c</sup> Conjugation frequency was measured as the number of transconjugates recovered per input donor cell.

<sup>d</sup> ND, not determined.
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 extracted with equal volumes of isopropanol saturated with 20× SSC (1× SSC is 0.15 M NaCl plus 0.0015 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 database for similarities.

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 cosmids bank of pAoF64/95 was constructed by cloning a Sau3AI partial digest of purified plasmid DNA into the cosmids vector pCP13b (Table 1) digested with BamHII and calf intestinal alkaline phosphatase (CIP). The clones were then packaged into λ using the Packaging 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 cesidom clone pMWS110 by the method of Dansken and Wanner (63). The kanamycin cassette of pKD4 was amplified using the following primers, which contained 5′ overhang sequences of traR: forward primer, 5′ GATCGCTGCGATCAGAGAG; reverse primer, 5′ GCCCGAGCTCATATGCCGGAATTCATATGGACGGTGACCTTCGCTCACTCATCGACATGACAGAAGTGTA. 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 NT4(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 traR primers containing EcoRI and SalI sites (underlined): traMleft, 5′ GATCCGAAGAGGGTGAAGGTGTGCTGCTGTTGC ACACTGCAGGAGCTGCTC 3′; traMrighth, 5′ GATCCAGTCAGTACGACATGTGACACCGCGACACACTGCCGAC 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 NT4(pAoF64/95) to yield NTL4(pAoF64/95:traMrighth:pVik107). The single-crossover mutation was confirmed by sequence analysis, and the mutated plasmid was genetically isolated by transformation into NTL4.

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 conjugal 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 conjugal 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 geneti-ically isolated the plasmids responsible for MOP catabolism by transforming strain NT6, a plasmidless derivative of A. tumefaciens strain NTL6 (Table 1), with total plasmid DNA isolated from each strain, selecting directly for MOP utilization. MOP-utilizing

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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 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 quorumone. 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 quorumone as growth proceeded in medium containing MOP (Fig. 2). Concomitant with the increasing levels of the acyl-HSL signal, donors grown...
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

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**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).
family Rhizobiales, 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. Moreover, while the other gene encoding a full-size TraR homolog in this region of the plasmid. The organization of the two Ti plasmids. The 10 genes 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. 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 user; + / −, very poor growth; −, no significant growth. The dotted lines indicate where the cosmid extends beyond the scope of the map.

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; agcA, 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. nirR, 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 user; + / −, very poor growth; −, no significant growth. The dotted lines indicate where the cosmid extends beyond the scope of the map.

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-
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 conjugal 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 quorumone. 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 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).
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 *traI*:*trb* 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).

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
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).

**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(pAoF64/95ΔtraR), 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(pAoF64/95ΔtraR, 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::traMpVik107), 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::traMpVik107, 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.
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 agrocinopine, the fourth member of the mannityl opine family (see Fig. S1 in the supplemental material). Consistent with this observation, pAoF64/95 lacks gacA, which codes for the enzyme that converts agrocin to MOP (69–71), and also the agt operon that encodes the agrocin 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 mocCE genes in pAoF64/95 are coupled with the 3′ end of the mot operon, and instead of two moc-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 traR operon, which codes for the type IV mating bridge, as well as TraR, the acyl-HSL synthase, is invariably directed and oriented divergently from repA of the repABC operon (reviewed in reference 7). Moreover, in Ti plasmids, the tra complex and the traR-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 pRL1J1 (79) and p42a (80). In these plasmids, the repABC complex and the two divergently oriented tra operons 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 pAoF64/95ΔtraR 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 conjuga-tive 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 conjuga-tive 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 traM 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 agrocinopines (48). Thus, the catabolic properties of...
pArA4 expand the range of the mannityl opines utilisable 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 sp., 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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Mannonipine-Inducible Transfer


