CHARACTERIZATION OF AN AGROBACTERIAL PLASMID INDUCIBLE FOR TRANSFER BY MANNOPINE AND EVOLUTION OF THE CORE REPLICATION AND TRANSFER FUNCTIONS OF REPABC PLASMIDS WITH CLASS I QUORUM-SENSING AND TRANSFER SYSTEMS

BY

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DISSERTATION

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Abstract

repABC plasmids are ubiquitous in the α-proteobacteria and are important to the biology of the bacteria that harbor them for several reasons. First, they can carry large amounts of DNA, thereby conferring a wide variety of important characteristics. Some of these traits are important for the biology of the bacteria that harbor them. For example the *repABC* plasmids in species of *Agrobacterium* can encode virtually all of the genes responsible for inducing crown gall tumors and hairy roots on susceptible plant hosts. These plasmids also encode the genes required for production by the plant, and utilization by the bacteria of unique carbon conjugates called opines. Similarly, *repABC* plasmids in species of *Rhizobium* confer nodulation and nitrogen fixation when the bacteria are in symbiosis with a suitable plant host. Second, the *repABC* replicons have a broad host-range, and a subset of these plasmids encode a conjugative transfer system allowing these biologically relevant elements to transfer between and among species of bacteria. Perhaps the best-studied transfer system of the *repABC* plasmids is the Class I system composed of a type four secretion system encoded by the *traI*/*trb* operon and a DNA metabolism system encoded by the two *tra* operons. These operons are regulated by a quorum-sensing system involving three proteins: TraR, TraI, and TraM. TraR directly activates the transfer regulon but needs its ligand, an acyl-homoserine lacone quorum-sensing signal synthesized by TraI, to be active. One additional component, TraM, binds to TraR directly and inactivates the quorum-sensing protein when the signal for transfer is absent.

The octopine-type Ti plasmids in *A. tumefaciens* strains 15955 and R10 are inducible for conjugative transfer by octopine because *traR* is the distal member of an operon inducible by the conjugative opine. However, a second non-functional allele of *traR*, called *trlR*, is present in the mannopine transport operon, an operon that is inducible by the opine mannopine. Based on the location and inducibility of *trlR* by mannopine, we hypothesized that there would be a functional allele of *traR* that is

similarly located in a mannopine-inducible operon and that mannopine would induce transfer of a plasmid in a wild-type isolate of *Agrobacterium*. To this end we characterized and analyzed a collection of mannopine-utilizing field isolates for the ability of mannopine to induce transfer. We found five such isolates. Further characterization of the mannopine-utilizing plasmids in these strains indicated that these plasmids all are highly related. We analyzed and sequenced one such element, pAoF64/95. First, pAoF64/95 is not a virulence element; it does not contain the genes for virulence or a T-region. Instead pAoF64/95 is an opine-catabolic plasmid and encodes all of the genes for utilization of three of the four mannityl opines- mannopine, mannopinic acid and agropinic acid- as well as the agrocinopines. Indeed, strains harboring pAoF64/95 can utilize these three mannityl opines and are also sensitive to agrocin 84, an indication that the strain can utilize the agrocinopine opines. Second, an otherwise plasmid-less strain harboring pAoF64/95 transfers the mannopine-utilizing trait to a recipient when grown with mannopine. Moreover, mutational analysis of *traR* and *traM* encoded by pAoF64/95 suggests that the functions of TraR and TraM as activator and antiactivator are conserved. Finally, the genes involved in Class I transfer of pAoF64/95 are not organized as they are in Ti plasmids. For all *repABC* plasmids with Class I transfer systems, the *traI*/*trb* operon is always adjacent and divergently oriented to the *repABC* operon. In the Ti plasmids, the *tra* region along with *traR* and *traM* are located distantly from the *trb*-*repABC* region and more often are located near the genes for uptake and catabolism of the conjugative opine. Additionally, in the Ti plasmids known to be conjugative, *traR* is invariably located in an operon inducible by the conjugative opine. This organization of the genes for conjugative transfer we call Group I organization. However, in pAoF64/95, like the Ri plasmids of *A. rhizogenes* and many plasmids in species of *Rhizobium*, the location of the *tra* genes is contiguous with the *trb*-*repABC* region and *traR* is monocistronic, an organization we name Group II.

Based upon these two modes of organization of plasmids with Class I transfer systems (Group I and Group II), we hypothesized that the component gene systems represent divergent evolutionary lineages. We assessed the evolution of the transfer, quorum-sensing, and replication and partition proteins and found that the quorum-sensing and transfer proteins form two clades that are consistent with the two modes of plasmid organization, indicating that the two organizational groups of plasmids are evolving divergently. Despite the obligatory linkage of the *repABC* operon with the *traI*/*trb* operon, the *repABC* proteins evolve independently of the transfer and quorum-sensing proteins. Moreover, while RepA and RepB coevolve, RepC evolves independently. Functional analysis indicates that TraR can dimerize and activate *tra* box-containing promoters of members within a clade, but not between clades. This is further evidence that proteins within, but not between clades are cross-functional. In contrast, the *oriT* regions are highly conserved and do not form two major clades. Consistent with the phylogeny, cloned *oriT* regions are processed and mobilized by members of either clade. We conclude that Group I and Group II plasmids diverged based upon where the cargo DNA is located and moreover that this divergence in organization extends to function.

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Chapter 1: Introduction and Literature Review

1.1 *repABC* plasmids are ubiquitous in the Rhizobiales and confer defining characteristics to the bacteria that harbor them

The α-proteobacteria are a diverse group of bacteria, that includes *Brucella*, which are pathogenic to humans and other animals; *Agrobacterium*, which are pathogenic to plants; *Rhizobium* and *Bradyrhizobium*, which are plant symbionts; and *Nitrobacter* and *Oligotropha*, organisms that grow both lithoautotrophically and chemoorganotrophically. Many members of the α-proteobacteria harbor secondary chromosomes or large plasmids that encode a *repABC* replication and partitioning system (21, 25, 188). This replication system is remarkable in that it can stably maintain large quantities of genetic information. Some of the *repABC* replicons are nearly as large as the primary chromosome of the bacteria that harbor them. Moreover, these *repABC* replicons often define the bacteria that harbor them. For example the virulence genes of some species of *Brucella* are encoded on a *repABC* secondary chromosome. Likewise, the virulence traits of *Agrobacterium* and the genes for plant symbiosis and nitrogen fixation found in some species of *Rhizobium* are encoded on large *repABC* plasmids (92, 93, 260).

The *repABC* genes generally are organized into a single operon that has a complex regulatory system (188). Both RepA and RepB are related to members of the Par family of proteins involved in segregation of chromosomes and other replicons (188). RepA and RepB are responsible for the stable maintenance and partitioning of the low copy-number plasmids. While RepC has no known homologues, it is required for and believed to initiate plasmid replication (6, 195). Moreover, the *oriV* lies within the coding sequences of *repC* [reviewed in (188)]. Most *repABC* replicons are maintained at a copy number of only one or two under normal growth conditions (25, 188).

The *repABC* plasmids often play a crucial role in the biology of the bacteria that harbor them. In addition to encoding the genes that define some of the bacteria that harbor these replicons, plasmids in the RepABC family are known to recombine and form cointegrates (122, 159, 255), thereby swapping or deleting gene cassettes and increasing the diversity of these replicons and the bacteria that harbor them. Furthermore, many of these plasmids also encode a conjugative transfer system responsible for transfer of the plasmid from one bacterium to another, thereby horizontally spreading the *repABC* plasmids and the traits they encode.

1.2 There are 3-4 classes of transfer systems

There are at least four classes of conjugative transfer systems associated with the *repABC* family of plasmids in the *Rhizobiales* [(89) and reviewed in (59)]. Many transfer systems are encoded by two functional units: mating pair formation (Mpf) and DNA metabolism (Dtr). In the most well-studied of these, the Class I transfer systems, the Mpf is an IncP-like, type four secretion system encoded by the 12 gene *traI*/*trb* operon (142). The Dtr system is composed of two divergently oriented operons, *traAFBH* and *traCDG*, and has a chimeric phylogeneny, encoding IncQ-like and IncP-like DNA metabolism genes and *oriT* sites (41, 73). The Class I system, which is a regulated by a quorum-sensing system, will be described in greater detail later.

The Class II transfer system is composed of a *virB*-like type four secretion system, called *avhB*, and an IncQ-like DNA metabolism system that is similar to the Class I Dtr system, but lacks three of the *tra* genes, *traF*, *traB* and *traH* (31). Class II systems are regulated by an *rtcA*/*rtcB*-like system (59, 183, 208). The inducing signal(s) for Class II transfer systems, if any, has not been determined.

The Class III transfer system, like the Class II system, lacks *traFBH* and additionally is missing *traC* and *traD* (59). While *traA* and *traG* homologues are still present in

the Class III system, *traG* is preceded by a putative *mobC* gene that encodes an additional *oriT*-nicking enzyme (59). Class III transfer systems seemingly lack a *trb*- or *virB*-type Mpf system and the plasmids are presumably not self-conjugative, but appear to be mobilizable via an Mpf system encoded elsewhere in the donor bacterium (59).

Class IV transfer systems, like the Class III system, lack the Mpf functions (89). The Dtr system includes a *mobC*-like relaxase gene similar to the Class III system and additionally contains a *mobZ*-like relaxase (89). Furthermore, Class IV Dtr systems contain a gene similar to the partitioning gene, *parA* (89). This system utilizes *trans*-encoded Mpf functions for mobilization to a recipient (89, 105).

1.3 The Class I conjugative transfer system is conserved in many megaplasmids in the Rhizobiales

The most well studied transfer systems in the Rhizobiales fall into Class I. The type IV Mpf system is encoded by the *traI-trbBCDEJKLFGHI* operon which may be transcribed as a single unit (142). However while only one promoter element has been described upstream of *traI*, there is evidence that there may be an additional transcriptional start site within the operon (10, 141). TraI encodes an acyl-homoserine lactone synthase that is involved in regulation of transfer by quorum-sensing (QS) (115, 142). Most of the remaining genes encode putative structural components of the Mpf system. For example, TrbC is a homologue of the IncP Mpf pilin precursor protein (63, 96). However, two of the proteins encoded by the operon, TrbJ and TrbK, are involved in entry exclusion and function in a synergistic fashion (37). While most of the genes are required for transfer, two genes are not. Mutations in *trbK* do not affect transfer frequencies while mutations in *trbI* decrease transfer frequency by 3-4 orders of magnitude (144). The *traI*/*trb* operon is located directly adjacent and divergently oriented to the *repABC* operon in all such plasmids examined to date.

The two divergently oriented *tra* operons, *traAFBH* and *traCDG*, and the origin of transfer (*oriT*), located in the intergenic region between the *traA* and *traC*, are involved in the Dtr functions of transfer. TraA, TraC, and TraD are believed to be components of the relaxosome that binds to and nicks at the *oriT* site (38). TraA is the strand transferase that nicks *oriT* and is an essential component of transfer (38, 73). TraC and TraD are not essential; however, these two proteins increase the frequency of both *oriT* processing and transfer (38). TraF, another essential protein, is the putative pilin processing protein required for cleaving the TrbC pre-pilin proteins to form the final pilin subunits (63, 73, 96). TraG is an essential protein in the Dtr system and is predicted to be the coupling factor that interfaces the relaxosome components with the T4SS encoded by the *trb* operon (73, 98). The TraB protein is a predicted amidohydrolase, and while it is not essential, it increases the efficiency of transfer (73). TraH is member of the lysozyme-like superfamily and is not required for transfer. Table 1.1 lists the proteins and their functions involved in Class I conjugative transfer and replication system.

1.4 A quorum-sensing system regulates transfer of Class I plasmids

The conjugation of plasmids with Class I transfer systems is directly regulated by a LuxR-type quorum-sensing (QS) system (83, 189, 264). The key protein, the activator TraR, binds to an inverted repeat sequence approximately 18 nt in length called a *tra* box and promotes transcriptional activation of all three operons of the transfer (*tra)* regulon (82, 83, 151, 268). There typically are at least three *tra* boxes, named *tra* box I, II, and III [(81, 83) and reviewed in (253)]. *tra* box I is located in the intergenic region between the *traAFBH* and *traCDG* operons, while *tra* box II is upstream of the *traI*/*trb* operon. *tra* box III lies between *tra* box II and the *repABC* operon [reviewed in (253)], and consistent with this location, TraR also influences plasmid copy number (143, 160, 181).

Table 1.1 The known or putative functions of *repABC* Class I transfer and replication proteins and DNA elements

Protein	Essential For Transfer[a]	Functional Class[b]	Function
TraI	Yes	Regulation	Acyl-homoserine lactone synthase, produces the acyl-homoserine lactone ligand of TraR
TrbB	Yes	Mpf	Involved in the Type IV secretion system
TrbC	Yes	Mpf	Putative pilin precursor
TrbD	Yes	Mpf	Involved in the type IV secretion system
TrbE	Yes	Mpf	Involved in the type IV secretion system
TrbJ	Yes	Mpf	Involved in entry exclusion
TrbK	No	Mpf	Involved in entry exclusion
TrbL	Yes	Mpf	Involved in the type IV secretion system
TrbF	Yes	Mpf	Involved in the type IV secretion system
TrbG	Yes	Mpf	Involved in the type IV secretion system
TrbH	Yes	Mpf	Involved in the type IV secretion system
TrbI	No, but increases frequency of transfer	Mpf	Involved in the type IV secretion system
TraR	Yes	Regulation	Quorum-sensing activator, binds to *tra* box DNA in an acyl-homoserine lactone dependent manner
TraM	No, TraM mutants are constitutive for transfer	Regulation	Anti-activator, antiactivates by binding to TraR
TraA	Yes	Dtr	Relaxosome component, strand transferase, recognizes and nicks the *oriT*
TraF	Yes	Dtr	Pilin processing protease
TraB	No, but increases frequency of transfer	Dtr	Predicted amidohydrolase
TraH	No	Dtr	Predicted lysozyme-like superfamily
TraC	No, but increases efficiency of transfer and *oriT* processing	Dtr	Putative relaxosome component
TraD	No, but increases efficiency of transfer and *oriT* processing	Dtr	Putative relaxosome component
TraG	Yes	Dtr	Coupling factor, interfaces the relaxosome with the type IV secretion system
TrlR	No	Regulation	Dominant-negative mutant of TraR, contains functional ligand and dimerization domains, but lacks the DNA binding domains
RepA	Yes	Plasmid replication/ partitioning	Plasmid partitioning

Table 1.1 (cont.)

RepB	Yes	Plasmid replication/ partitioning	Plasmid partitioning
RepC	Yes	Plasmid replication/ partitioning	Plasmid replication
DNA elements			
tra box	NA	Regulation	Inverted repeat sequence, TraR binding sequence
oriT	NA	Dtr	Origin of transfer, conserved DNA sequences that is recognized by the relaxosome and nicked by TraA

[a] NA, not applicable.

[b] Dtr, DNA metabolism; Mpf, mating pair formation.

TraR, like most LuxR homologues, requires an acyl-homoserine lactone (acyl-HSL) quorum-sensing signal to activate transfer (189, 262, 264). The acyl-HSL signal, invariably N-3-oxo-octanoyl-L-homoserine lactone (AAI, 3-oxo-C8), is synthesized by the product of *traI*, the first gene in the *traI*/*trb* operon (115, 142). TraI is expressed at a very low but detectable level and small amounts of the acyl-HSL are always produced (82, 115). When the population density has reached a critical threshold and enough of the acyl-HSL has accumulated, TraR is able to bind its ligand, form stable dimers and activate transcription of the *tra* regulon (194, 268, 269).

The quorum-sensing system in many of the plasmids in the Rhizobiales has one additional component, TraM. In several tested systems in which conjugation is strongly repressed, mutations in *traM* result in constitutive transfer (81, 114, 191). TraM binds to TraR, thereby inactivating it and targeting TraR for proteolysis (30, 43, 116, 152). TraM thereby serves as an antiactivator and inhibits premature activation of the transfer genes by TraR in the absence of the conjugative signal (44, 81, 82, 114). When the conjugative signal is present the levels of activated TraR

overcome the effects of TraM. TraM is expressed at relatively high levels and, at least among some of the large plasmids of *Agrobacterium*, expression of TraM increases when TraR is active (81). Such a feedback loop could serve to decrease the expression levels of the *tra* regulon when the population density drops (81).

1.5 Conjugative plasmids in the family *Rhizobiaceae* and their regulation

Members of the Rhizobiaceae, including the genera *Rhizobium* and *Agrobacterium*, harbor Class I *repABC* family plasmids that are known to transfer to a recipient. For some of these plasmids, the regulatory mechanisms and inducing signals have been described. We will first discuss conjugative plasmids in species of *Rhizobium* and then discuss the more intensely-studied transfer systems of plasmids in species of *Agrobacterium*.

1.5.1 Plasmids of *Rhizobium* spp.

Strains of *Rhizobium* spp. are known for their ability to induce root nodules in plants, some of which fix nitrogen. Genes for induction of the nodules (*nod*) and nitrogen fixation (*nif*/*fix*) usually are carried by *repABC* family Sym plasmids. Several of these plasmids also carry the Class I conjugative transfer and quorum-sensing genes.

1.5.1.1 pRL1JI- a plasmid that expresses transfer functions in the presence of a suitable recipient

Rhizobium leguminosarum bv. *viciae* strain A34 establishes symbiosis with legumes and provides the plant with forms of fixed nitrogen (60). The genes required for nodulation and nitrogen fixation are encoded on a large *repABC* plasmid called pRL1JI (60). Intriguingly this plasmid also encodes a Class I conjugative transfer and QS system. The *traI* gene encodes an acyl-HSL synthase that produces (3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL) and the TraR ortholog responds to

the TraI-produced 3-oxo-C8 HSL signal (44). $TraM_{pRL1JI}$ reduces TraR mediated transcription in the absence of the appropriate signal (44).

Transcription of the monocistronic $traR_{pRL1JI}$ is activated by a second, in this case orphaned, LuxR protein called BisR, also encoded by pRL1JI (257). BisR, in turn, activates transcription in response to a second acyl-HSL, N-(3-hydroxy-7-cis-tetradecenoyl)-L-homoserine lactone (3-OH-C14:1-HSL) (257). Interestingly, strain A34 does not produce this second signal. Rather induction of transfer requires a recipient strain that produces the quormone (44, 149, 257). Therefore an acyl-HSL signal produced by the recipient induces transfer of pRL1JI from the donor.

1.5.1.2 p42a- a plasmid that expresses transfer functions at a high constitutive level

Rhizobium etli strain CFN42 harbors six plasmids (93). One of these, p42a, is self-conjugative at high frequencies (18). Expression of the *tra* regulon and concomitant transfer of p42a is positively regulated by two LuxR QS regulators, TraR and CinR (243). Both TraR and CinR positively regulate expression of *traI* (243). Furthermore, $TraI_{p42a}$ is required for transfer and produces 3-oxo-C8-HSL (243). While expression of *cinR* is dependent upon TraI, expression of *traR* is constitutive (243). The authors propose that 3-oxo-C8 HSL is the ligand for both CinR and TraR and that these two LuxR quorum-sensing proteins act in parallel to directly activate the three operons of the *tra* regulon (243).

Interestingly TraM of p42a, unlike orthologs in other well-studied systems, does not influence transfer (243). Furthermore, expression of $traM_{p42a}$ was not detected (243). Considering that transfer of p42a is constitutive and not strongly dependent on population size, it is likely that this phenotype results from a lack of a functional TraM.

1.5.2 Plasmids of *Agrobacterium* spp.

1.5.2.1 A bacterium that induces neoplasias on plants

Species of *Agrobacterium* cause crown gall and hairy root diseases on susceptible plants. *A. tumefaciens* was first isolated from crown gall tumors and described as the causative agent of the disease in 1907 by Smith and Townsend (215). The plant neoplasias are the result of true transformation of the plant cells and do not require the continued presence of the bacteria (15, 16, 256).

The crown gall tumors themselves synthesize novel low molecular weight carbon compounds, now known as opines (146, 147, 170). Furthermore, the types of opines present in the tumor are not dependent upon the type of plant, but rather upon the strain of *A. tumefaciens* that induced the tumor (90, 186). The observation that bacteria are only required for the initial inducing step of infection and that crown galls produce opines specific to the strain of bacterium inducing the tumor led to the proposal that a genetic component of the bacterium is transferred to the plant during infection (186).

1.5.2.2 Large plasmids are the infectious component

Two lines of evidence further supported the idea that a genetic component of the bacterium is involved in tumorigenesis. The first involved loss of virulence of a particular isolate of *A. tumefaciens*, strain C58, when the strain was grown at elevated temperatures (99). Second, Kerr demonstrated that the virulence trait could be transferred to an avirulent strain of *Agrobacterium in planta* (128, 129). These two sets of experiments suggested that the virulence trait is associated with an epigenetic element and that this element is transmissible between bacteria. In 1974, the Schell group correlated virulence of agrobacterial strains with the presence of large plasmids and conversely found that the avirulent strains examined

were missing these elements (246, 261). Furthermore, Watson *et. al.* demonstrated that transfer of the virulence trait between bacteria *in planta* involved transfer of the plasmid from the pathogen to the recipient (251). The transmissible plasmid responsible for the induction of crown galls was called the tumor inducing (Ti) plasmid.

1.5.2.3 Ti plasmid: virulence and T-DNA

The Ti plasmids characterized to date all are large *repABC* elements, generally in excess of 150 kb. They encode both a segment of DNA (T-region) that is transferred to the plant during the infection (33) and all of the *trans*-acting machinery that mediates such transfer. During infection, the T-region is nicked; displaced, presumably by strand replacement; and processed into a single stranded intermediate called the T-strand (2, 120, 217, 239). The T-strand and several *trans*-encoded virulence proteins are transferred into the wounded plant cell through the *virB*-encoded type four secretion system (T4SS). Once there, the T-strand translocates into the plant nucleus where it integrates in a semi-random fashion into the plant genome (135, 237).

The type four secretion system that transfers the T-strand to the plant cell is a modified conjugative transfer system encoded by the virulence (*vir*) regulon of the Ti plasmid. The *vir* regulon is composed of several operons, including the *virB* and *virD* operons. The T4SS consists of 12 proteins, VirB1-VirB11 and VirD4. The VirB proteins are most closely related to those of the IncN plasmids that are involved in conjugative transfer between bacteria (140, 192, 211). VirD4, another essential component of virulence, is distantly related to the coupling protein TraG of RP4 (98, 138, 140, 145, 193). This family of proteins is thought to interface the relaxosome components with the type four secretion system (98). The *virB2-virB11* genes are essential for pilus formation and virulence. *virB1* is not required for virulence, although a *virB1* mutant is severely attenuated (12, 80).

1.5.2.4 Oncogenes

The T-regions from various Ti plasmids can be divided into two functional regions: those that are highly homologous between all Ti plasmids and those that are more variable (32, 53, 107). The conserved regions, which confer the oncogenic properties to the plant host (49, 53, 68, 107), encode three important genes. The first gene, *iaaM*, codes for tryptophan 2-monooxygenase which converts tryptophan into indole-3-acetamide (IAM) (236, 247). The second gene, *iaaH*, codes for indole-3-acetamide hydrolase which hydrolyzes IAM into indole-3-acetic acid (IAA), a phytohormone in the auxin class (117, 206, 235). A third gene, *ipt,* encodes dimethylallylpyrophosphate (DMAPP) transferase (also called isopentenyl transferase, *ipt*), which converts DMAPP and AMP into isopentenyl-AMP, a product that can be converted by the host plant cell into various cytokinins (1, 5, 19). In normal plant cells auxins and cytokinins act together to regulate cell growth and division, and when produced together at high levels in transformed plant cells, cause the uncontrolled proliferation of the transformed cells characteristic of crown gall disease.

1.5.2.5 Opine synthesis

The variable segments within the T-regions (32, 107) encode functions that are responsible for synthesis of the opines (*ops*) characteristic of a tumor induced by a given strain of *A. tumefaciens* (17, 107, 113, 121, 137, 201, 207, 258). Among the first opines identified were nopaline and members of the octopine family: lysopine, histopine, octopine, and octopinic acid (13, 91, 161, 162). Both the octopine and nopaline families are imine derivatives of an α-ketoacid. Nopaline is a conjugate of α-ketoglutarate and arginine, while the members of the octopine family are imines of pyruvic acid and the amino acids arginine (octopine), lysine (lysopine), and histidine (histopine) (Table 1.2). The four-member mannityl family of opines- agropine, mannopine, mannopinic acid, and agropinic acid- were later detected,

Table 1.2 Opines and opine families and their chemical characteristics[a]

Chemical Bond	Substitution on the Imine Group	Opine Family	Opine Name	Moiety A	Moiety B
Sugar Phosphodiesters	NA[b]	Agrocinopines	Agrocinopine A	Sucrose	Arabinose
			Agrocinopine B	Fructose	Arabinose
			Agrocinopine C	Sucrose	Glucose
			Agrocinopine D	Glucose	Glucose
Imine linkage	Sugars	Mannityl Opines	Agropine (lactone)	Mannose	Glutamine
			Mannopine	Mannose	Glutamine
			Mannopinic acid	Mannose	Glutamate
			Agropinic acid (lactam)	Mannose	Glutamate
		Chrysopine	Deoxy-fructosyl-glutamine	Deoxy-fructose	Glutamine
			Chrysopine (lactone)	Deoxy-fructose	Glutamine
			Isochrysopine (lactam)	Deoxy-fructose	Glutamine
			Deoxy-fructosyl-5-oxyproline (lactam)	Deoxy-fructose	Proline
	α-Ketoacids	Octopine	Octopine	Pyruvic acid	Arginine
			Octopinic acid	Pyruvic acid	Ornithine
			Lysopine	Pyruvic acid	Lysine
			Histopine	Pyruvic acid	Histidine
		Heliopine	Heliopine	Pyruvic acid	Glutamine
			Heliopine lactam	Pyruvic acid	Glutamine
		Nopaline	Nopaline	α-Ketoglutarate	Arginine
			Nopalinic acid	α-Ketoglutarate	Ornithine
		Succinamopine	Succinamopine	α-Ketoglutarate	Asparagine
			Succinamopine lactam	α-Ketoglutarate	Asparagine
			Leucopine	α-Ketoglutarate	Leucine
			Leucopine lactam	α-Ketoglutarate	Leucine
		Cucumopine/ Mikimopine	Cucumopine/ Mikimopine	α-Ketoglutarate	Histidine
			Cucumopine/ Mikimopine lactam	α-Ketoglutarate	Histidine
		Ridéopine	Ridéopine	α-Ketoglutarate	Putracine
			Ridéopine lactam	α-Ketoglutarate	Putracine

[a] This table is a modification and expansion of Table 1 in (56).

[b] NA, not applicable.

purified, and characterized as being imines of mannose and glutamine, or mannose and glutamate (Table 1.2) (78, 226, 228). While most opines are imine derivatives of either a sugar or α-ketoacid and an amino acid, the four opines of the agrocinopine family, agrocinopine A+B and agrocinopines C+D, are composed of two sugars linked by phosphodiester bonds (Table 1.2) (65, 197). To date, over 20 different opines have been identified that fall into nine families (Table 1.2).

1.5.2.6 Ti plasmid- catabolism of opines and the opine concept

There is a correlation between the opines produced by tumors and the ability of the strain of *Agrobacterium* that induces the tumor to utilize specific opines (148). For example, strains that induce crown gall tumors that synthesize nopaline also utilize nopaline (148). Such correlations are a function of the Ti plasmid; while there are many different kinds of opines, any given Ti plasmid encodes both the genes for synthesis by the plant and utilization by the bacterium of only two or three families of opines. For example, the T-regions of the octopine-type Ti plasmid code for the genes for synthesis by the tumors of all four members of the octopine family and all four members of the mannopine family of opines (50, 67). Encoded elsewhere on this megaplasmid are the genes for both uptake and utilization by the bacterium of these two families of opines (39, 57, 76, 109, 133, 134, 155, 205, 244). Moreover, other bacteria in the rhizosphere generally cannot utilize opines [reviewed in (56, 232)]. The ability of the bacteria to engineer the plant to produce opines from plant metabolites and the ability of the bacteria to utilize the opines as a source of carbon and/or nitrogen led to the opine concept (229) and the related genetic colonization theory (204). These concepts essentially state that bacteria genetically engineer their plant hosts to provide a nutritional niche that provides a selective advantage when in competition with other rhizosphere microflora (204, 229).

The opine concept was extended to include other species of *Agrobacterium*, including *A. rhizogenes*, the causative agent of hairy root disease. Like *A.*

tumefaciens, virulence of isolates of *A. rhizogenes* is due to the oncogenes encoded on a large *repABC* plasmid called the root inducing (Ri) plasmid (254). These plasmids are functionally similar to the Ti plasmid; however, based on plasmid homology and virulence functions, the Ri plasmids and Ti plasmids represent two evolutionary lineages (255). Similar to crown gall tumors induced by *A. tumefaciens*, hairy roots induced by *A. rhizogenes* produce opines. For example, tumors induced by *A. rhizogenes* strain A4 produce all four mannityl opines and agrocinopines A+B (185, 234). Tumors induced by other strains of *A. rhizogenes* produce mikimopine, which is an imine derivative of α-ketoglutarate and histidine (Table 1.2) (47, 48, 118, 119). In summary, as with the Ti plasmids, the Ri plasmids encode the genes for opine synthesis in transformed plant cells and the genes for opine catabolism by the bacterium (185, 214).

Opine-like compounds have been identified in plant root nodules induced by certain species of *Rhizobium* (230, 231). These compounds, called rhizopines, are nodule-specific compounds that can be catabolized by the bacterium that induced the root nodule. The genes encoding both the synthesis and catabolism of the rhizopines are located on the large *repABC* Sym plasmid responsible for plant symbiosis and nitrogen fixation (172, 199). However, the genes for synthesis of the rhizopines are not integrated into the plant genome. Rather, they are expressed in bacteroids in the root nodule and the compounds are synthesized from host plant metabolites and made available to the free-living rhizobia on the surface of the nodule (173).

1.5.2.7 Ti plasmids are transferred to recipient strains in plant tumors

While Kerr demonstrated that the Ti-plasmid could be transferred to a recipient strain *in planta* (128, 129), his early experiments attempting to demonstrate such transfer *ex planta* in laboratory media failed (129). Two lines of evidence suggested that a compound from the plant neoplasias induced transfer of Ti plasmids between

bacteria. First, Ti plasmids were transmissible only when the crosses were done in the environment of the plant tumor. Second, transfer efficiency increases over the course of crown gall development, which suggests a compound unique to the tumor is accumulating and thereby increasing transfer efficiencies (130).

1.5.2.8 Opines induce conjugative transfer of Ti plasmids

Following these studies, two groups determined that growth with a particular opine, octopine, stimulated transfer of an octopine-type Ti-plasmid to an avirulent recipient (88, 130). There is strong specificity to the particular opine that induces transfer. For example, tumors induced by strains harboring the octopine-type Ti plasmid also produce the four mannityl opines, but none of these opines induces transfer of this Ti plasmid (187). In a similar fashion, nopaline-type Ti plasmids encode catabolism of the nopaline family of opines, as well as agrocinopines A+B. Ellis and colleagues demonstrated that while nopaline does not induce transfer of such plasmids, agrocinopines A and B are the conjugative opines (64). The agropine-type Ti plasmid, pTiBo542, which encodes genes for utilization of succinamopine, all four members of the mannityl opine family, and agrocinopines C+D, is inducible for transfer only by agrocinopine C and D (64).

1.5.2.9 Genes involved in conjugative transfer are organized based on plasmid type

As noted previously, the *traI*/*trb* operon involved in the Mpf functions of transfer is invariably located adjacent and divergently oriented to the *repABC* operon. The *traAFBH* and *traCDG* operons encoding the Dtr functions are always divergently oriented with respect to each other and the *oriT* is located in the ca. 200 bp intergenic region between these two operons. However, as first noted by Moriguchi *et. al.*, the location of the Dtr genes with respect to the *traI*/*trb-repABC* locus varies based on plasmid type (171). In the case of the large plasmids of *Rhizobium* and the

Ri plasmids of *A. rhizogenes* the *tra* and *trb* operons are contiguous. In these plasmids *traR* is monocistronic and located downstream of the last gene in the *traI*/*trb* operon, while *traM* is located distal to *traR* and near the last gene of the *traA* operon (Figure 1.1). However, in the Ti plasmids and at least one plasmid of *Rhizobium* spp., the two *tra* operons along with *traR* and *traM* are not closely linked to the *traI*/*trb* operon (Figure 1.1). Furthermore, at least where transfer has been demonstrated, *traR* is always in an operon, the expression of which is controlled by an external signal (84, 177, 178, 190).

1.5.2.10 Conjugative transfer of Ti plasmids is inducible by an opine because *traR* is a member of an opine-inducible operon

traR of Ti plasmids is always located in an operon that is controlled by the conjugative opine. Under most circumstances *traR* is expressed at a relatively low basal level (82, 177, 178, 190). However, the presence of the conjugative opine induces transcription of this operon, including *traR* (82, 177, 178, 190). Once *traR* is translated, the QS activator is made in sufficient quantities that it titrates the effects of the antiactivator, TraM. Free TraR can then bind the quormone, form stable dimers, and activate expression of the *tra* regulon. In this manner, the presence of the conjugative opine is responsible for inducing conjugative transfer.

1.5.2.11 Examples of opine-controlled conjugative transfer

When this study began, only four opines were known to induce conjugative transfer of plasmids harbored by strains of *Agrobacterium*: octopine, agrocinopines A+B, agrocinopines C+D and nopaline [reviewed in (72)].

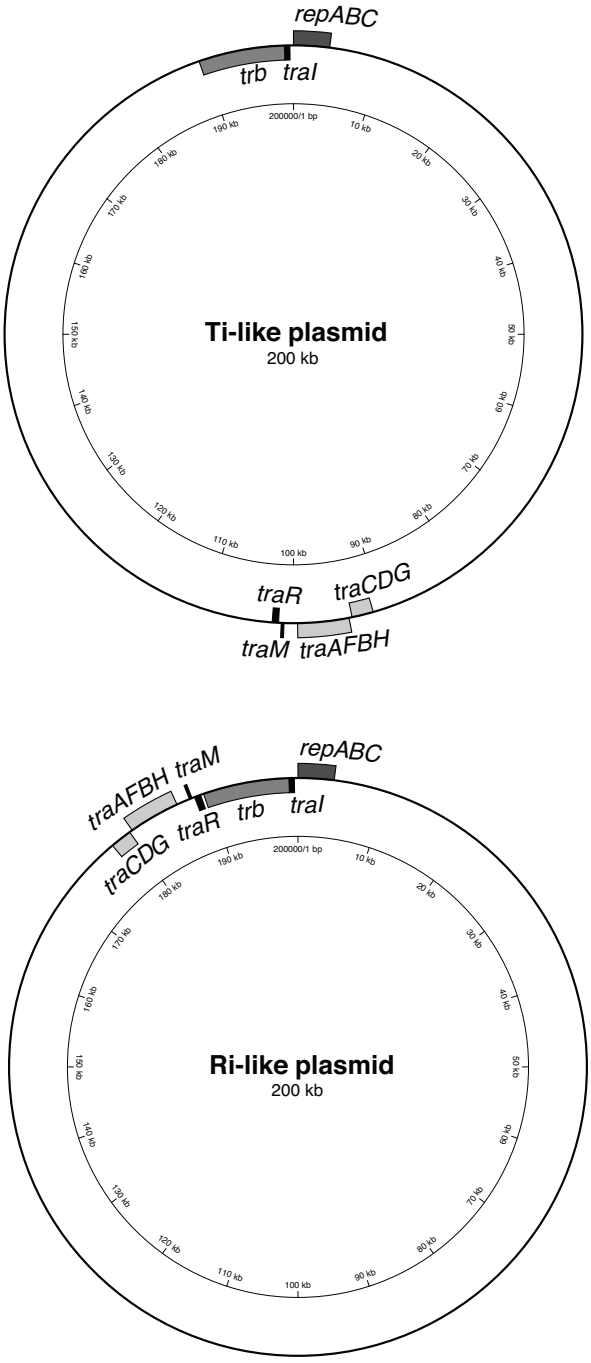


Figure 1.1 There are two organizational patterns of *repABC* plasmids with Class I transfer systems. The genes involved in quorum-sensing regulation are colored black while the DNA metabolism functions and mating pair formation systems are pictured in light and medium grey respectively and the *repABC* operon is pictured in dark grey.

1.5.2.11.1 Octopine-type Ti plasmids

Several independent isolates of *A. tumefaciens* harbor essentially identical octopine-type Ti plasmids (266). pTi15955 and pTiR10, which confer utilization of octopine and all four of the mannityl opines, are the most well studied of these elements, and transfer of these Ti plasmids is induced by octopine (82, 187). For both pTi15955 and pTiR10, *traR* is the distal member of the *occ* operon that encodes transport and catabolism of octopine (Figure 1.2) (82, 85). This operon is regulated by OccR, a LysR-type activator that responds to octopine; when the opine is present, OccR binds the opine and activates the *occ* operon, and *traR* is expressed yielding levels of the activator that can overcome the effect of the antiactivator, TraM (82, 84, 97).

1.5.2.11.2 pTiC58 and agrocinopines A+B

Classic nopaline-type Ti plasmids such as pTiC58 confer utilization of both nopaline and agrocininopines A+B. However, only agrocinopines A and B induce transfer of this plasmid (64). Two closely linked and divergently oriented operons encoded by pTiC58 are inducible by the agrocinopines: *acc*, which encodes the agrocinopine uptake and catabolism functions, and *arc*, of which *traR* is a member (Figure 1.2) (190). A FucR-like repressor, called AccR, represses both of these operons, and repression is relieved in the presence of the agrocinopines A and B (8, 190). As with the octopine-type Ti plasmids, expression of *arc* results in levels of TraR that titrate TraM and activate the *tra* regulon (190).

1.5.2.11.3 pTiBo54 and pTiChry5 and agrocinopines C+D

As described above, conjugative transfer of the agropine plasmid is induced by agrocinopines C and D (64). A second related plasmid, pTiChry5, induces tumors that produce a set of three mannityl opine-type compounds called chrysopine, deoxy-fructosyl glutamine (Dfg) and isochrysopine, as well as agrocinopines C+D

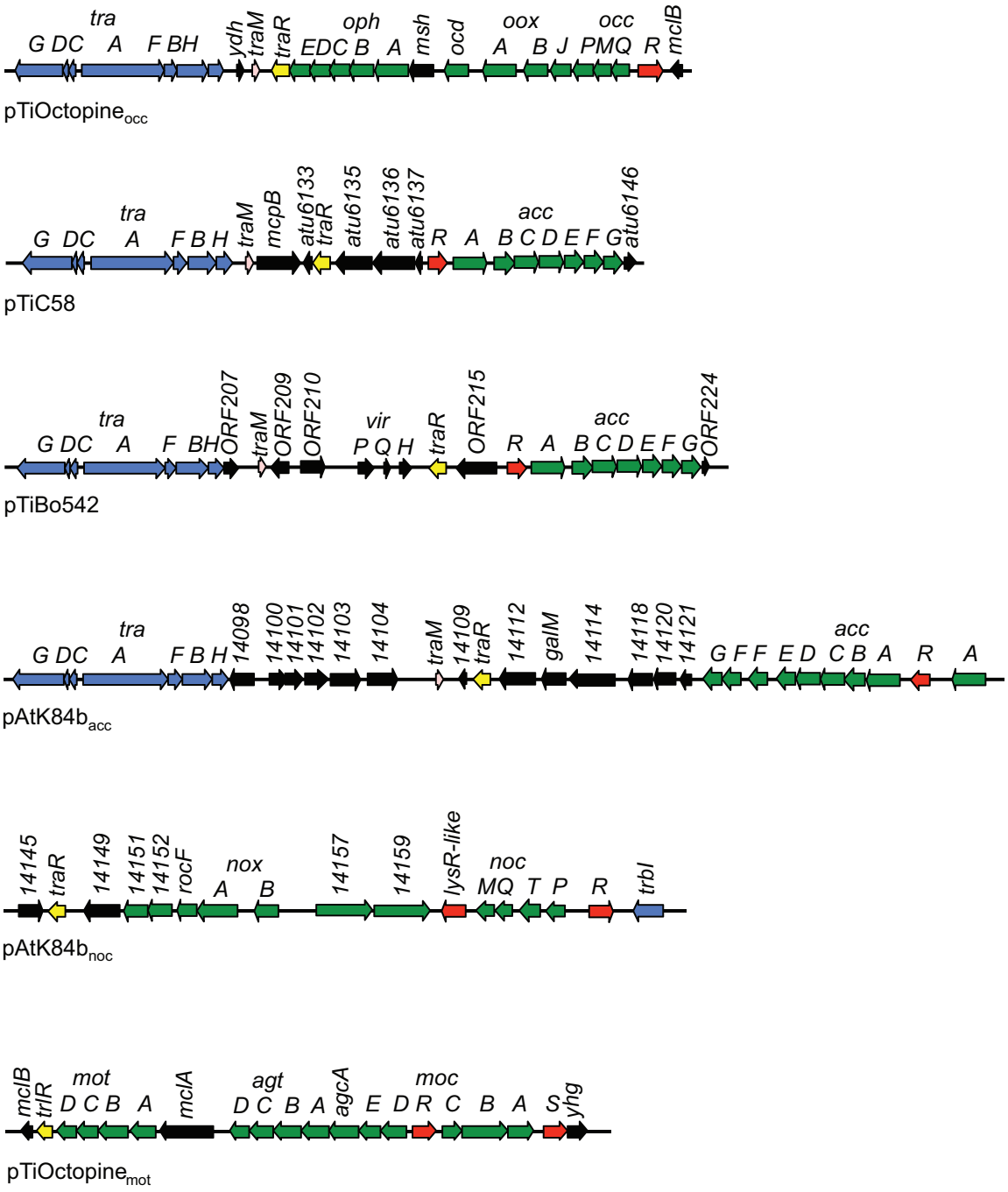


Figure 1.2 Opines induce transfer because *traR* is a member of an opine-inducible operon. The genes for DNA metabolism and mating pair formation are colored in blue, while *traR* and *traM* are colored in yellow and pink respectively. Genes for opine uptake and catabolism are pictured in green while genes that are known or putative regulators of the opine catabolic operons are in red. All other genes are pictured in black.

(36, 177, 249). As with pTiBo542, only agrocinopines C and D induce transfer of pTiChry5 (177). Sequence analysis of both pTiBo542 and pTiChry5 revealed that *traR* is located in an operon that is related to the *arc* operon of pTiC58 (Figure 1.2) (177). Moreover both plasmids encode a repressor that is orthologous to AccR from pTiC58 (177).

1.5.2.11.4 pAtK84b- some plasmids are "cheaters" that do not encode virulence, but code for opine utilization and transfer to a recipient

The opine concept states that virulent strains of *Agrobacterium* spp. and perhaps related organisms engineer their environment to produce a niche in which the bacteria enjoy a selective advantage (204, 229). Ti plasmids are the classic example of this model, where the plasmid encodes the functions for virulence and production by the plant and catabolism by the bacteria of specific opines. However, non-pathogenic strains of *Agrobacterium* spp. are easily isolated from plant tumors, and many of these can utilize opines (127, 163). Furthermore, most of these strains contain large plasmids, some of which contain regions of homology with Ti plasmids (163). *Agrobacterium radiobacter* K84, an avirulent strain, harbors a large *repABC* plasmid, pAtK84b, that confers utilization of nopaline and agrocinopines A+B but does not encode any virulence functions (40, 213). In addition, pAtK84b encodes all the genes involved in a Class I QS-regulated conjugative transfer system (213).

1.5.2.11.5 pAtK84b- some plasmids have more than one copy of *traR* and more than one opine induces transfer

Plasmid pAtK84b is self-transmissible and, unlike Ti plasmids, transfer is induced by both nopaline and agrocinopine A+B (64, 178). Sequence analysis revealed that pAtK84b encodes two copies of *traR* (178, 213). The first, $traR_{acc}$, is located in an operon similar to the *arc* operons of pTiC58, pTiBo542, and pTiChry5 (Figure 1.2) (178). Transcription of $traR_{acc}$ is inducible by growth with agrocinopines A+B, but

not nopaline (178). Furthermore, transfer of a *traR*$_{acc}$ mutant is no longer inducible by agrocinopines but is still inducible by nopaline (178). The second copy of *traR*, *traR*$_{noc}$, is located within the *noc* operon responsible for transport and utilization of nopaline (Figure 1.2) (178). The *noc* genes as well as *traR*$_{noc}$ are inducible by nopaline but not the agrocinopines (178). Likewise, a *traR*$_{noc}$ mutant only transfers when cells are grown with agrocinopines A+B (178). A double *traR*$_{acc}$*traR*$_{noc}$ mutant fails to transfer pAtK84b when grown with either opine (178).

1.6 The octopine-type Ti plasmids encode a mutant allele of *traR* inducible by mannopine

While octopine serves as the conjugative opine for the octopine-type Ti plasmids, pTi15955 and pTiR10, these plasmids also encode a second allele of *traR* associated with the genes for uptake and catabolism of the mannityl opines (179, 267). This allele, called *trlR*, is the distal gene of the mannopine transport operon, *motABCDtrlR*, and encodes a frame-shifted mutant of the QS activator that contains functional ligand-binding and dimerization domains but lacks the C-terminal DNA binding domain (Figure 1.2) (179, 267). Growth of strains harboring either of these Ti plasmids with mannopine induces transcription of *trlR* but does not induce transfer (179, 267). Indeed, growth of strains harboring these Ti plasmids with both octopine and mannopine results in transfer at lower frequencies compared to growth with octopine alone (179, 267). Restoring the frame-shift allowed the protein to activate a TraR-dependent promoter (267). TrlR binds to AAI, similar to TraR, and purified TrlR inhibits TraR-dependent DNA binding and activation (28). Moreover, TrlR forms inactive heterodimers with TraR$_{octopine}$ (28, 179, 267).

1.7 Purposes of this study

This thesis began with two objectives that stem from the observation that the octopine-type Ti plasmids encode a nonfunctional allele of TraR that is inducible by MOP.

1. Are there wild-type plasmids in which MOP can induce conjugative transfer?
2. If so, does this induction by MOP involve a functional allele of *trlR* that is associated with the mannopine transport operon?

We screened a number of wild-type isolates of *Agrobacterium* spp. for MOP-inducible conjugative transfer of the MOP catabolism trait and found five isolates that are capable of transfer when grown with the mannityl opine. We genetically isolated and sequenced one of the plasmids, pAoF64/95, responsible for MOP-dependent transfer. This plasmid does not encode any virulence genes or T-region, which is consistent with fact the parental isolate is not pathogenic. Furthermore this plasmid encodes a full set of Class I transfer and QS genes organized in one contiguous unit with a monocistronic copy of *traR*. This organization is similar to that of the Ri plasmids of *A. rhizogenes* and other plasmids harbored by spp. of *Rhizobium* (Figure 1.1). This plasmid also carries a full set of genes required for the transport and catabolism of three of the four mannityl opines. However, *traR* is not associated with the genetic loci involved in uptake and catabolism of the mannopine. So while we identified plasmids in which MOP serves as the conjugative opine, the organization of the relevant genes is not like that of *trlR* of the octopine-type Ti plasmids.

We then addressed regulation of transfer of pAoF64/95 using standard molecular genetics. Consistent with the well-studied systems, TraR and TraM are the activator and antiactivator of conjugative transfer.

We were intrigued by the observation that in the large *repABC* plasmids with Class I QS transfer systems the genes involved in transfer and replication are organized either as one contiguous unit, as seen in pAoF64/95, most of the plasmids of *Rhizobium*, and the Ri plasmids of *A. rhizogenes*, or as two separate units as in the Ti plasmids of *A. tumefaciens* (Figure 1.1). We hypothesized that the proteins of the QS and transfer systems from plasmids with an Ri-like organization would be more closely related to their orthologous proteins from other plasmids with an Ri-like organization. Moreover we hypothesized that the same proteins from plasmids organized like Ti plasmids would be more closely related to their orthologs from plasmids with similar genetic organization. Considering that the *traI*/*trb* operon is invariably located adjacent to the *repABC* operon we also examined the evolution of RepA, RepB, and RepC to see how their evolutionary histories compare with the evolutionary histories of the transfer and QS proteins. This led to the third aim of this thesis:

> 3. Evolution of the Rep, QS, and Class I transfer systems of plasmids in the Rhizobiales.

We used both *in silico* and *in vivo* approaches to assess several of the conjugative transfer and QS proteins, along with RepA, RepB, and RepC to determine their evolutionary relationships. First, despite close linkages, the replication system does not coevolve with the transfer and quorum-sensing systems. Second, the genes of the QS and transfer systems generally coevolve within a particular plasmid. Moreover, the regulatory and transfer genes of the plasmids that are organized like Ti plasmids form one clade, while plasmids that have the QS and transfer genes organized into one contiguous unit, as seen in the Ri plasmids and plasmids of *Rhizobium* spp. form a separate clade. The two organizational types of the *tra* regulon represent both evolutionary and functional divergences of the Class I QS and transfer systems of the large plasmids in the Rhizobiales.

Chapter 2: Quorum-Dependent Mannopine-Inducible Conjugative Transfer of an *Agrobacterium* Opine-Catabolic Plasmid

2.1 Notes and acknowledgments

This chapter was adapted from a paper published in the *Journal of Bacteriology* entitled "Quorum-dependent mannopine-inducible conjugative transfer of an *Agrobacterium* opine-catabolic plasmid," March 2014, Volume 196, Pages 1031-1044, with authors Margaret E. Wetzel, Kun-Soo Kim, Marilyn Miller, Gary J. Olsen, and Stephen K. Farrand. Kun-Soo Kim originally screened the 11 field isolates for mannopine-inducible conjugative transfer. I repeated these experiments with three of the isolates that were found to be inducible for transfer by MOP. All transfer values located in Table 2.2 were the result of Kun-Soo's experiments. Marilyn Miller sent us the collection of wild-type field isolates (the Corvallis isolates) collected by Larry Moore's laboratory at Oregon State University. She did the initial strain characterizations, including opines utilized, host plant, location, tumorigenicity, agrocin sensitivity, and biovar determination. I repeated the tumorigenicity, agrocin sensitivity, and biovar determinations for a select set of the isolates. Gary J. Olsen helped with analyzing plasmid sequences and wrote a program to illustrate the map of the plasmid. Finally we dedicated this paper and chapter to the late Larry Moore.

2.2 Summary

The Ti plasmid in *Agrobacterium tumefaciens* strain 15955 encodes 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 and is located in an operon that is inducible by the opine mannopine (MOP). Based on these findings we predicted 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 encodes genes for catabolism of MOP, mannopinic acid (MOA), agropinic acid (AGA) and the agrocinopines. The plasmid additionally encodes 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.

2.3 Introduction

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 (29, 112, 259)]. The transferred strand subsequently is integrated into the plant cell nuclear genome [reviewed in (87)] and results in production of plant growth hormones responsible for the crown gall tumors [reviewed in (266)]. Additionally, the integrated T-DNA encodes the genes for synthesis by the neoplasias of novel compounds called opines [reviewed in (232, 266)]. 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 bacterium that induced the tumor [reviewed in (232)]. Remarkably, the Ti plasmid also encodes 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 (72, 74).

Ti plasmids generally code for uptake and catabolism of two or three of the eight known opine families (35, 55, 56, 72). 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 (72). For instance pTiC58 codes for the uptake and catabolism of two opine types, nopaline and agrocinopines A+B, but only the agrocinopines induce plasmid transfer (64). The conjugative opines, however, do not directly induce transfer of pTiC58; rather they control transcription of *traR*, which codes for the quorum-sensing (QS) activator TraR (8, 190). TraR directly activates the Ti plasmid *tra* and *trb* operons responsible for DNA metabolism and mating pair formation respectively (42, 71, 83, 142-144).

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+B uptake and catabolism operon (*acc*) (190). The divergently oriented *acc* and *arc* operons are both transcriptionally repressed by the opine-responsive repressor, AccR (8, 190). When the agrocinopines are available, repression by AccR is lifted and *traR*, as part of the *arc* operon, is expressed (190). 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) (189, 262, 264). This acyl-HSL quormone is synthesized by TraI, which is encoded by the first gene of the Ti plasmid *trb* operon (115, 142). QS-dependent induction of transfer is additionally

modulated by TraM, an anti-activator that interacts with TraR, thereby inhibiting activation of the *tra* regulon (81, 114, 116).

Although in most cases only one opine serves as the conjugative signal, this is not always the case. For instance, *A. radiobacter* strain K84 harbors an opine-catabolic megaplasmid, pAtK84b, that is inducible for transfer by two opine types; agrocinopine A+B and nopaline (64, 178). This plasmid encodes 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+B (178). 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 (178).

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, mannopine, mannopinic acid and agropinic acid [reviewed in (72)]. These plasmids encode two alleles of *traR*. One, $traR_{oct}$, is a member of an operon that is activated by octopine, and this opine induces conjugative transfer (82). The second called *trlR*, is the terminal gene in the mannopine (MOP) transport operon, *motABCDtrlR*, located in the mannityl opine catabolic region of these Ti plasmids (179). Expression of this operon, including *trlR*, is inducible by MOP, but not by octopine (179, 267). 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 (179, 267). The protein product can dimerize with the full-sized, functional TraR, induced by octopine, thereby inhibiting octopine-inducible conjugative transfer (28, 179, 267).

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.

2.4 Materials and methods

2.4.1 Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 2.1. The mannopine-utilizing field isolates of *Agrobacterium* spp. were obtained from the laboratory of the late Dr. Larry Moore, Oregon State University, and have not been previously described in the literature. Methods for collection of the field tumors, isolation of bacteria, determination of opine catabolism, and pathogenicity testing all were described in Moore, Chilton and Canfield (168).

2.4.2 Media and growth conditions

Strains of *Escherichia coli* were grown in L broth (Fischer Scientific), and in SOB or SOC media (100). Strains of *Agrobacterium* spp. were grown in L broth (Fisher Scientific), MG/L (20), or on Nutrient Agar (Difco). Stonier's medium (218) was used for agrocin 84 sensitivity assays and AB (20) and AT (233) 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 carbon sources. Mannopine and mannopinic acid were the kind gifts of Dr. Yves Dessaux, Institut des

Table 2.1 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*	(150)
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	*hsd*R514(r_k^-, m_k^+) *gln*V(*sup*E44) *try*T (*sup*F58) *lacY*1 or Δ(*lacIZY)*6 *gal*K2 *galT*22 *metB*1 *trpR*55	Promega
S17-1 λpir	Tp^r Sm^r *recA thi pro hsdR-M*$^+$ RP4::2-Tc::Mu::Km Tn7 λpir	(212)
Plasmids		
pAgK84-A1	Agrocin 84 producer	(75)
pArA4	MOP^+ MOA^+ AGA^+	J. Tempe'
pCP13/B	Deletion derivative of pCP13; Tc^r	(45)
pKD46	Amp^r; λ Red helper plasmid	(46)
pSRKGm	Gm^r; IPTG-inducible expression vector	(131)
pViK107	Kan^r; promoterless *lacZY*; *pir* dependent	(124)
pWM91	Amp^r *sacB*	(164)
pZLR4	Gm^r; indicator for detection of acyl-HSL	(209)

[a] Abbreviations: MOP, mannopine; MOA, mannopinic acid; AGA, agropininic acid; AGR, agropine; $TraI^+$, production of AAI; Amp^r, ampicillin resistance; Gm^r, gentamicin resistance; Kan^r, kanamycin resistance; Rif^r, rifampicin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.
[b] OC, our collection.

Sciences du Végétal, Gif-sur-Yvette, France. Antibiotics were used at the following concentrations in μg/ml: rifampicin 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.

2.4.3 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 in Moore, Kado and Bouzars (169): 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.

2.4.4 Virulence assays

The Moore laboratory described the virulence properties of all wild-type strains as tested on tomato plants (Table 2.2). We repeated these assays with the five MOP-inducible strains using the stem-wound inoculation method on tomato plants essentially as described previously (67, 180).

2.4.5 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. Washed cells were inoculated into liquid AB mannitol (ABM), AB + 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,

Table 2.2 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] Wild-type isolates listed here all utilize mannopine and were obtained from Larry Moore, Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR.
[b] Opines that were tested are MOP, mannopine; NOP, nopaline; OCT, octopine.
[c] Conjugation frequency is measured as transconjugates recovered/input donor cell.
[d] ND, not determined.

volumes were removed, a 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.

2.4.6 Conjugative transfer efficiency

Conjugative transfer of the *Agrobacterium* plasmids to strain C58C1RS (Table 2.1) was determined using the drop plate method (10, 74, 191). Transconjugants were selected for using opine catabolism or resistance to an appropriate antibiotic and donors were counterselected using rifampicin and streptomycin.

2.4.7 Detection and quantification of AAI

AAI was extracted from culture supernatant essentially as described previously (74, 154, 209). For chromatographic analysis, the concentrated samples were spotted on a C_{18} reverse-phase thin-layer chromatography plate and separated using methanol-H_2O (60:40 vol/vol) (74, 209). For quantification, 5 µl volumes of a two-fold dilution series of each sample were spotted in a grid pattern onto a TLC plate. The samples were allowed to dry, 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) (74, 209), and incubated overnight at 28°C. The plates were dried, scanned, and ImageJ (version 1.44o; National Institute of Health [http://imageJ.nih.gov/ij]) (196) 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 (74).

2.4.8 Agrocin 84 sensitivity assays

A modified version of the procedure of Hayman and Farrand (102) was used to assess sensitivity to agrocin 84. Briefly, two colonies of the agrocin 84 producer strain, NT1(pAgK84-A1) (Table 2.1), were suspended in 0.25 ml of 0.9% NaCl and 15 µl of this suspension were spotted onto the center of Stonier's agar (1.5% agar) plates (218) and incubated at 28°C for three days. The producer strain was killed by exposure of the plate to chloroform vapors for 15 minutes. 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.

2.4.9 Isolation of mannityl opines from tumors

Crude preparations of the mannityl opines were isolated from plant tumors as described previously (56, 177). Briefly, tomato tumors induced by *A. 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 minutes. The mixture was ground with mortar and pestle and the solids were removed by decanting the mixture over cheese-cloth. The remaining particulate matter was removed by centrifugation and the tumor extract was sterilized by passage through a 0.22 μm filter.

2.4.10 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 electrophoresis (HVPE) using an acetic acid-formic acid buffer with a pH between 1.7 and 1.9 according to the methods described previously (56, 67, 185). The opines were visualized using the alkaline silver nitrate reagent as described previously (20, 67, 241).

2.4.11 Mannityl opine utilization studies

Growth on solid medium was assessed by visual inspection of strains inoculated on AB agar media with MOP or MOA as the sole source of carbon. The plates were observed daily over a seven-day period. Growth was compared to appropriate positive and negative controls and recorded as -, 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 media (34). Sterilized tumor extract prepared as described above was added to 2× AT media 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 five 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.

2.4.12 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 three hours, 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 (109).

2.4.13 Preparation and purification of plasmid DNA

Small-scale plasmid isolation from *E. coli* was done by the alkaline lysis methods of Sambrook and Russell (203). 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 (263). In all other cases, large- and small-scale isolations of plasmids from *Agrobacterium* strains were performed as described by Hayman and Farrand (103). 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× SCC 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).

2.4.14 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 (69, 70) and clean sequences were assembled using PHRAP (51, 95). After final assembly, open reading frames were called using Glimmer [(version 3.02) (52, 202); http://www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi]. We used BLAST to search the NCBI non-redundant 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.

2.4.15 DNA manipulations

Agrobacterium strains were transformed using the freeze-thaw method (106) and *E. coli* was made competent using the calcium chloride method (203). Occasionally, plasmids were mobilized from *E. coli* into *Agrobacterium* using a filter-mating technique (74). An overlapping cosmid bank of pAoF64/95 was constructed by cloning a Sau3AI partial digest of purified plasmid DNA into the cosmid vector pCP13b (45) 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 digest and the ends of appropriate inserts were sequenced using primers complementary to vector sequences.

2.4.16 Construction of mutant strains

All PCR reactions 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 (46). The kanamycin cassette of pKD4 was amplified using the following primers that contained 5′ overhang sequences of *traR*: Forward primer- 5′ GTGGACGGTGACCTTCGCTCACTCATCGACATGACAGAAGTGTAGGCTGGAG CTGCTTCG 3′, Reverse Primer- 5′ CTACAGCAGGCCGTGGTCCTTGGCGATCGCGACGAG GTGCATATGAATATCCTCCTTAGT 3′. The PCR product was transformed into *E. coli* (pKD46, pMWS110) following the published protocol (46) and alleles of *traR* in which the cassette was inserted in 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′ AGCTCGCGAGGACTTGAATACCCGG 3′ and traRcheckdwn- 5′ GATCGCTGCGATCAGAGAGCACCG 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 (164). This resulting plasmid was transformed into *E. coli* S17-1/λpir and the plasmid was transferred into NTL4(pAoF64/95) by filter mating (74). 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 megaplamid, 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 (124). 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′ GCCGGAATTCATATGGACGGTGACCTTCGCTC 3′) and traRpSRKGmantisense (5′ CGCGAATTCGGATCCTACAGCAGGCCGTGGTCCT 3′). The *traM* gene was separately amplified by PCR using the following primers: traMpSRKGmsense (5′ GCCCGAGCTCATATGAGCGACGTGAACTCGTCTG 3′) and traMpSRKGmantisense (5′ GCGGAGCTCGGATCCTCAATCACCGACTTCGGGGGC 3′). The *traR* and *traM* genes were directionally cloned into pSRKGm (131) 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 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 at a final concentration of 1 mM to the cultures.

2.4.17 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.

2.5 Results

2.5.1 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 2.2). 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 2.2).

All but two of the 11 Corvallis isolates classified as biovar 2 agrobacteria. Six of the isolates induced tumors on at least one of three plant hosts tested (Table 2.2). Of the nine isolates tested, five were susceptible to agrocin 84, an antiagrobacterial antibiotic to which susceptibility is conferred by particular plasmid-encoded opine catabolic systems (Table 2.2) (132, 227). Detailed growth studies of the five MOP-inducible isolates indicated that all of them also utilize mannopinic acid (MOA) and agropinic acid (AGA), but none utilize agropine (AGR) (Figure 2.1 A). Consistent with this pattern, none of these five isolates expressed detectable levels of mannopine cyclase activity (Figure 2.1 B).

2.5.2 Mannopine catabolism and its inducible transfer is 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 (Figure 2.2 A). Given the possibility that one or more of the isolates harbors more

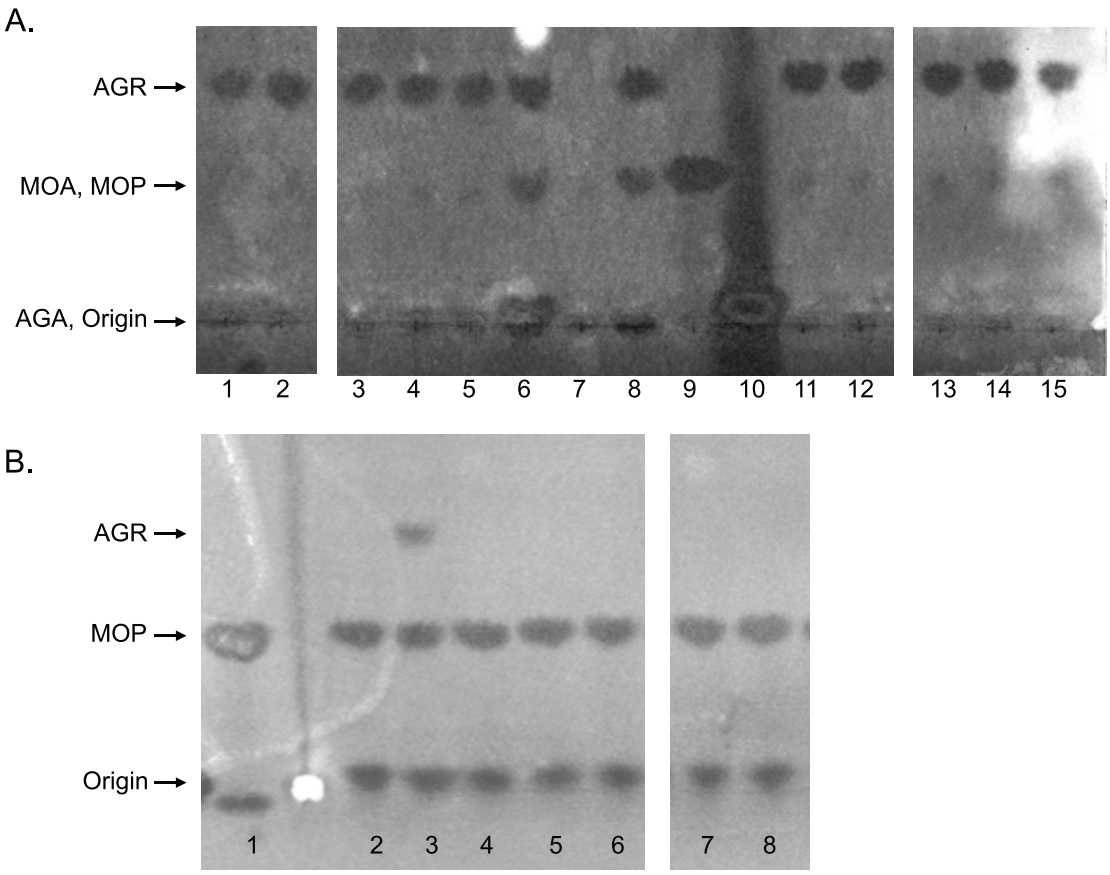


Figure 2.1 The Corvallis isolates and their transformants can utilize MOP, MOA, and AGA, but not AGR. A. Strains were cultured in AB minimal medium supplemented with extracts from tumors induced by strain 15955. Following growth, the cells were removed and the culture supernatants were analyzed for the mannityl opines by high-voltage paper electrophoresis (HVPE) all as described in materials and methods section 2.4.10. Supernatants are from cultures of 1, F64/95; 2, F265/93; 3, J62/95; 4, J84/95; 5, M200/94; 6, Uninoculated; 7, 15955; 8, NTL6; 11, NTL6(pAoF64/95); 12, NTL6(pAoF265/93); 13, NTL6(pAoJ62/95); 14, NTL6(pAoJ85/95); 15, NTL6(pAoM200/94). Lanes 9 and 10 contain mannopine and fructose standards respectively. **B.** The Corvallis isolates were analyzed for MOP cyclase activity as described in materials and methods. Opines were separated by HVPE and visualized using an alkaline silver nitrate stain. Lanes contain the reaction products from incubation with: 2, NTL4(pTiC58); 3, 15955; 4, M200/94; 5, J84/95; 6, J62/95; 7, F265/93; and 8, F64/95. Lane 1 contains a MOP standard.

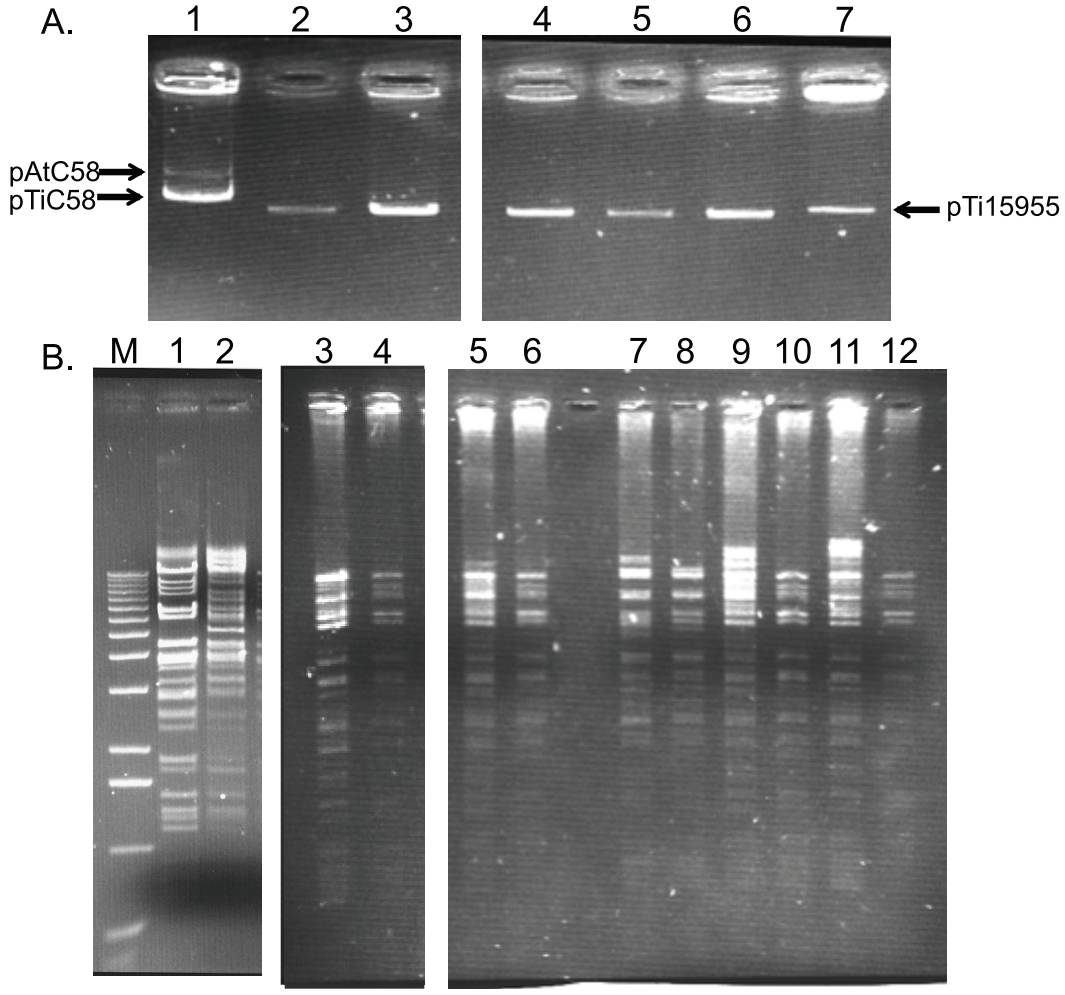


Figure 2.2 Isolates that transfer MOP catabolism harbor a related plasmid. **A.** Plasmid profiles of the MOP-inducible isolates. Plasmid DNA was isolated from the wild-type isolates. Lanes contain DNA from 1, NTL4 (pTiC58); 2, F64/95; 3, F265/93; 4, J62/95; 5, J84/95; 6, M200/94; and 7, 15955. The standards 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, digested with BamHI, and the fragments were separated on a 0.8% agarose gel. Lanes contain: 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).

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 2.1), with total plasmid DNA isolated from each strain, selecting directly for MOP utilization. MOP-utilizing transformants were isolated in each case, and individual colonies from each transformation tested also utilized MOA and AGA, but not AGR (Figure 2.1 A).

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 (Figure 2.2 B, compare odd- and even-numbered lanes). Moreover, the fragment patterns of plasmids from the five different transformants were very similar to each other (Figure 2.2 B, 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.

2.5.3 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 (82, 177, 178, 189, 262). 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 N-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL) (Figure 2.3). 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, as well as 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, assayed for growth by viable 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 (Figure 2.4). 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 (Figure 2.4). Concomitant with the increasing levels of the acyl-HSL signal, donors grown with MOP transferred pAoF64/95 at increasing frequencies as growth continued (Figure 2.4). Donors grown with mannitol failed to transfer the plasmid at a detectable level at any stage of growth tested.

2.5.4 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 (Figure 2.5). We also constructed an overlapping cosmid clone bank of the plasmid (Figure 2.5). Like most if not all large plasmids in the 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.

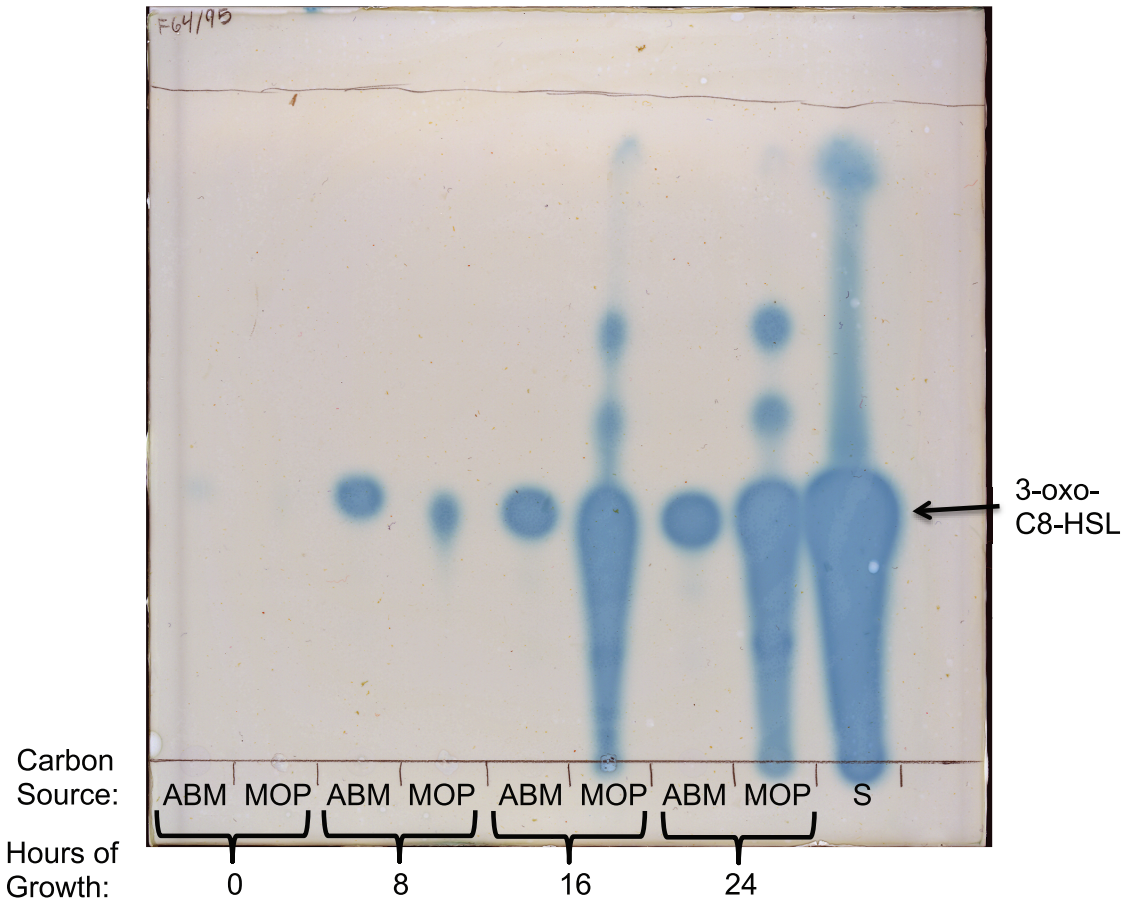


Figure 2.3 Production of AAI by F64/95 is induced by growth with MOP. Cultures grown in AB minimal medium supplemented with 0.005% yeast extract and either mannitol (ABM) or mannopine (MOP) as the primary carbon source were sampled approximately every eight hours for 24 hours. Samples of each culture were extracted with ethyl acetate, and the extracts were assayed for acyl-HSLs by reverse-phase thin-layer chromatography as described in material and methods section 2.4.7. S, authentic N-3-oxo-ocatanolyl-L-homoserine lactone (Sigma).

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, encoding 22 genes organized in six groups, that is closely related to regions of Ti plasmids known to confer catabolism of MOP, MOA and AGA (Figures 2.5 and

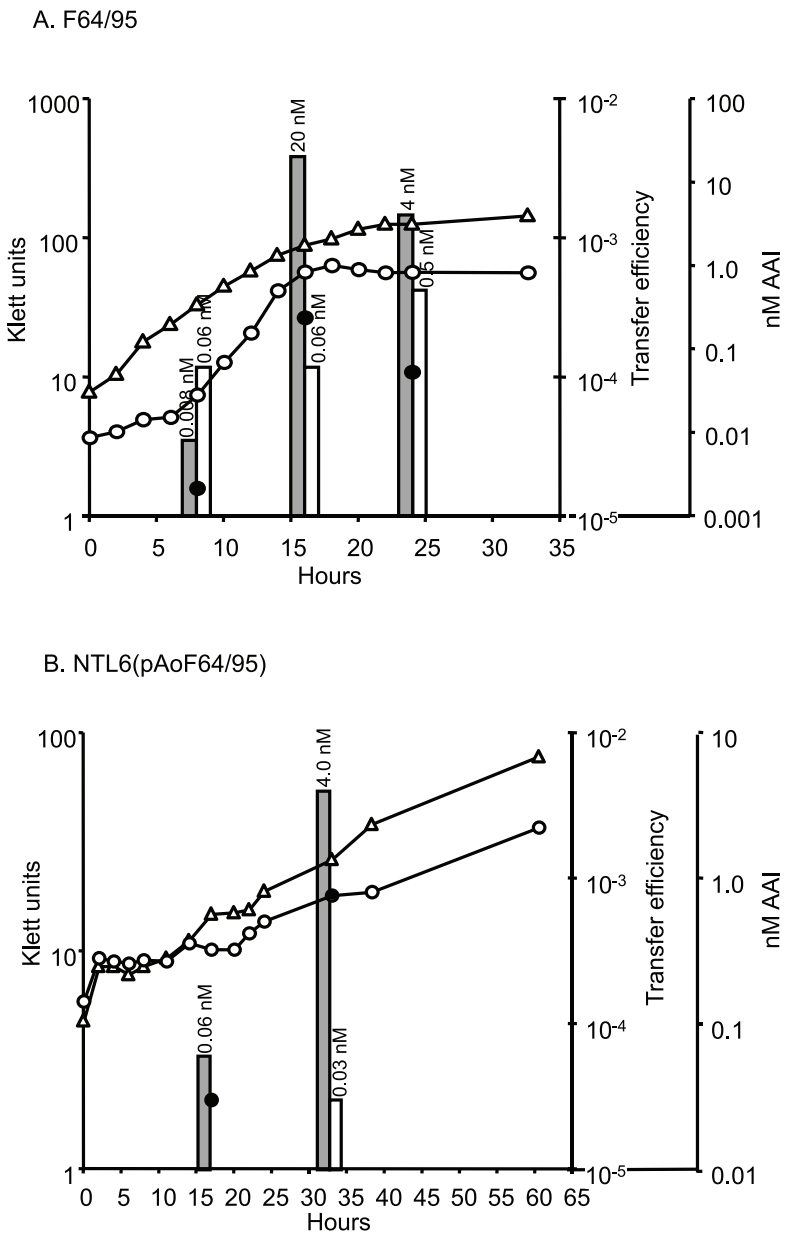


Figure 2.4 Growth with mannopine induces production of AAI and conjugative transfer of pAoF64/95. Strains F64/95(**A**) and NTL6(pAoF64/95) (**B**) were grown in AB medium with 0.005% yeast extract and mannitol (open triangles and open bars) or MOP (open circles and grey bars). Growth was followed turbidometrically using a Klett colorimeter. At approximately eight-hour 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. Transfer frequency is expressed as transconjugates recovered per input donor cell.

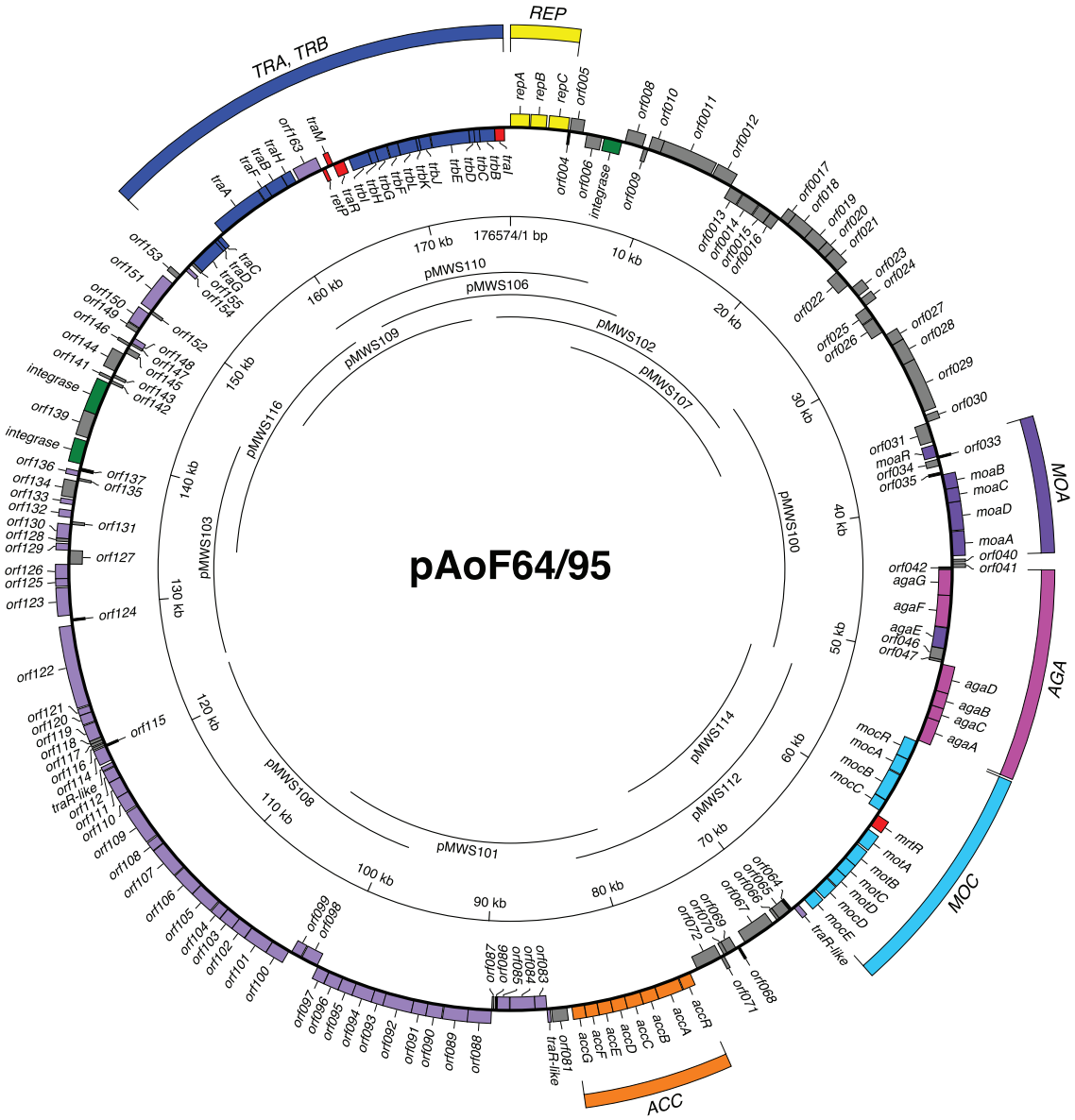


Figure 2.5 Physicogenetic map of pAoF64/95. The complete 176,574 bp sequence of pAoF64/95 was determined and the sequence was annotated as described in materials and methods section 2.4.14. Significant open reading frames are shown as boxes, with those on the outside oriented in the clockwise direction and those on the inside oriented in the 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

Figure 2.5 (cont.)
Ri plasmids, pRi1724 and pRi2659; grey, other genes that have a returned hit in the BLAST database; black, ORFs with no significant similarities in the data bases. The extent of the inserts in each cosmid in the ordered library is represented by the arcs within the rings. The annotated sequence is available in GenBank accession number JX683454.

2.6). The plasmid does not contain *agcA*, encoding catabolic mannopine cyclase (54, 108, 110), or the four-gene *agt* operon required for agropine uptake (109, 110), 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 (Figure 2.6). 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-structure differ (Figure 2.6). 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 sized TraR homolog in this region of the plasmid. Moreover, while the *moc* region of pTi15955 encodes two closely related putative regulatory genes, *mocR* and *mocS*, (123, 133) the corresponding region of pAoF64/95 encodes only one such gene we call *mocR* (Figure 2.6).

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 one of three cosmids, pMWS112, pMWS114, or pMWS115 (Figure 2.5 and 2.6 B). Restriction enzyme and sequence analysis showed that these three cosmids overlap the putative *moc* and *mot* genes and part of the AGA transport and

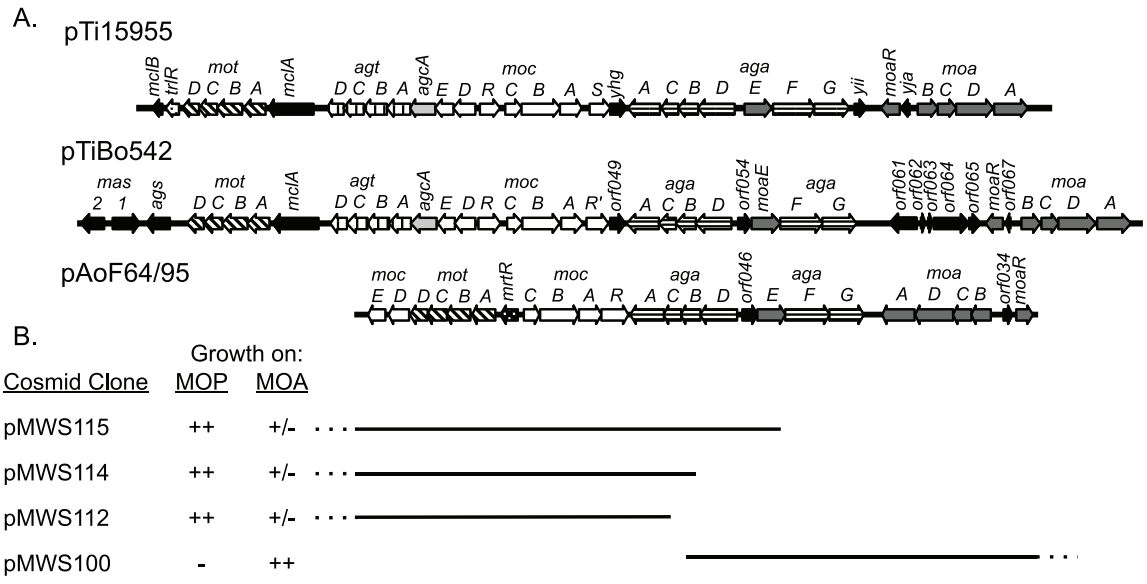


Figure 2.6 The mannityl opine catabolism region of pAoF64/95 is related to those of other *Agrobacterium* plasmids. A. The regions coding for the catabolism of the mannityl opines from two Ti plasmids, pTi15955 and pTiBo542, and from pAoF64/95 are aligned. Orthologous genes and gene systems are depicted 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,* a frame-shifted allele of *traR* in pTi15955 (179, 267). **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 their ability to grow on AB minimal agar containing either MOP or MOA as the sole carbon source. ++, growth as good as 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.

catabolism operons (Figure 2.6 B). In the second strategy, we transformed a cosmid, pMWS100, in which the insert overlapped the putative MOA catabolic region and part of the AGA transport and catabolism operons, but not the MOP catabolism region (as depicted in figure 2.6 B), into strain NTL4 selecting for

resistance to tetracycline. We screened these transformants for their ability to utilize MOP and MOA. NTL4(pMWS100) utilized MOA but not MOP (Figure 2.6 B), which is consistent with our bioinformatics predictions of this cosmid clone.

Plasmid pAoF64/95 encodes a second putative opine catabolic region, *acc*, mapping between coordinates 77 and 84 kb (Figures 2.5 and 2.7). On certain Ti and Ri plasmids orthologous loci code for uptake and catabolism of the agrocinopines, a family of sugar phosphodiester opines (65, 132), as well as susceptibility to agrocin 84, the unique antiagrobacterial antibiotic (132, 227). Of the five strains with pAoF64/95-like plasmids four were described to us 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 giving cloudy zones of growth inhibition (Figure 2.7 B). Strain NTL4(pAoF64/95) gave similar, although often more defined, zones of growth inhibition (Figure 2.7 B).

2.5.5 pAoF64/95 encodes a Ti plasmid-like conjugative transfer system and all of the components of a TraR-TraI quorum-sensing regulatory system

By bioinformatic analysis, pAoF64/95 encodes a single identifiable conjugative transfer system, and this system is virtually identical in gene content and operonal organization to those of other Ti, Ri, and opine catabolic plasmids (Figure 2.8). The 12-gene *trb* operon, encoding an IncP-like type IV secretion system, is adjacent to and oriented divergently to the *repA* gene of the replication operon (Figures 2.5 and 2.8). The two DNA-processing operons, *traAFBH* and *traCDG* are adjacent to and divergently oriented with respect to each other, although the latter encodes an additional putative small open reading frame, *orf155*, not present in the *traCDG* operons of other Ti plasmid-like systems (Figure 2.5). 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 (38, 41) (Figure 2.9).

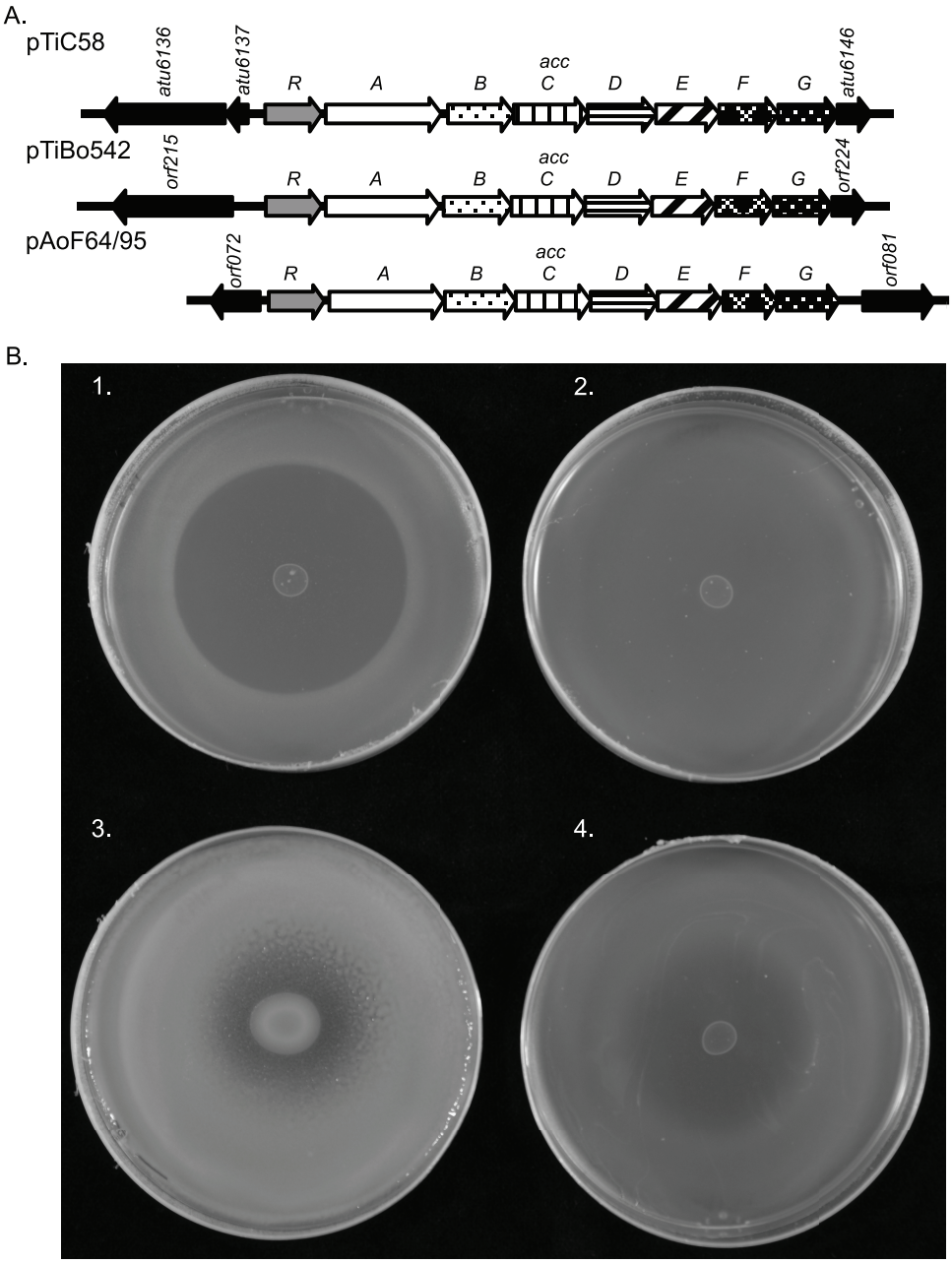


Figure 2.7 pAoF64/95 encodes a locus related to the agrocinopine A+B catabolism operon. A. Alignment of the *acc* operons from pTiC58, pTiBo542 and pAoF64/95. *accR* of pTiC58 and pTiBo542 encode the repressor that coregulates opine catabolism and conjugative transfer. Genes encoding orthologous functions are shown with the same fill pattern while genes not directly involved in catabolism and transport are black. **B.** Agrocin 84 sensitivity assays. Strains were assessed for sensitivity to agrocin 84 as described in materials and methods section 2.4.8. 1, C58; 2, NTL4; 3, F64/95; 4, NTL4(pAoF64/95).

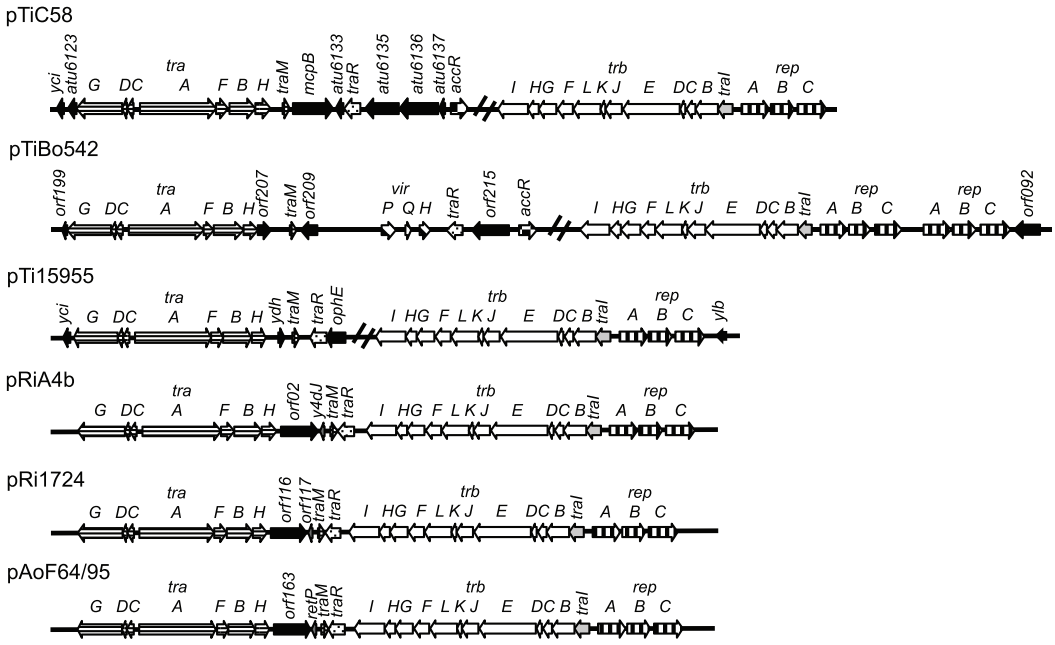


Figure 2.8 The *tra* and *trb* operons of Ti, Ri, and opine catabolic plasmids are conserved. The regions coding for DNA metabolism (*tra*) and mating-pair formation (*trb*) from three Ti plasmids (pTiC58, pTiBo542 and pTi15955) two Ri plasmids (pRiA4b and pRi1724), and pAoF64/95 were aligned. Orthologous genes and gene systems are depicted as arrows with identical fill patterns. Genes known to be involved in regulation of Ti plasmids transfer are: *traM,* ; *traR,* ; *traI*, . The plasmid replication genes are denoted *rep*. In pTiC58 and pTiBo542 the product of *accR*, , represses the *acc* and *arc* operons (8). The two Ri plasmids and pAoF64/95 encode a small helix-turn-helix, *xre* family gene, . Novel genes not characterized to date are filled in black. The double slash lines on the Ti plasmids indicate where the *tra* and *trb* regions are separated on those plasmids.

Figure 2.9 The *oriT* region of pAoF64/95 is strongly conserved with those of two Ti plasmids, pTiC58 and pTiR10. Nucleotides identical in all three regions are black with white lettering. Nucleotides conserved in two of the three regions are grey with white lettering. The *nic* site of pTiR10 (38) is indicated by an arrowhead.

The conjugative transfer region also encodes 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 (Figure 2.8). Moreover, the general locations and orientations of these three genes in pAoF64/95 closely resemble those in other known conjugative elements. *traI*, which encodes the acyl-HSL synthase (115), 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 certainly not a member of a plasmid-specific opine-regulated operon (Figure 2.8).

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 encode *traI*, produced low but detectable levels of the signal, while the strain harboring pMWS109, which maps to the same region but does not overlap *traI*, failed to produce the acyl-HSL (Figure 2.10).

2.5.6 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 (42, 71, 83, 142-144). 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 (Figure 2.11 A). Moreover, these donors failed to produce elevated levels of the acyl-HSL signal under any growth conditions tested

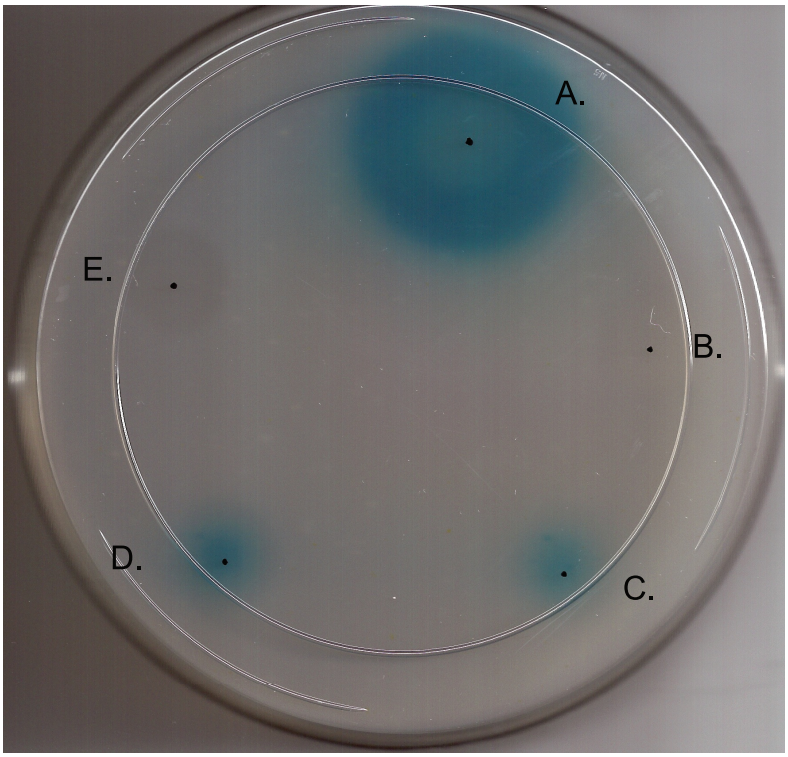


Figure 2.10 Cosmid clones of pAoF64/95 with inserts that contain *traI* code for production of an acyl-HSL. Overnight cultures of NTL4 harboring cosmid clones of pAoF64/96 that overlap the *tra*/*trb* region were grown in L broth. The cells were removed by centrifugation and 40 µl of the culture supernatants were spotted directly onto the surface of a soft agar layer of AB medium supplemented with mannitol and X-gal containing the indicator strain, NTL4(pRKLH4141) (153). Supernatants are from overnight cultures of: **A.** NTL4(pAoF64/95::*traM*pVik107), an AAI overproducing strain; **B.** NTL4(pMWS109), (*traI*$^{-}$); **C.** NTL4(pMWS106), (*traI*$^{+}$); **D.** NLT4(pMWS110), (*traI*$^{+}$); and **E.** NTL4.

(Figure 2.11 B). 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 pre-grown with MOP as the primary carbon source and expression of the recombinant *traR* was induced with

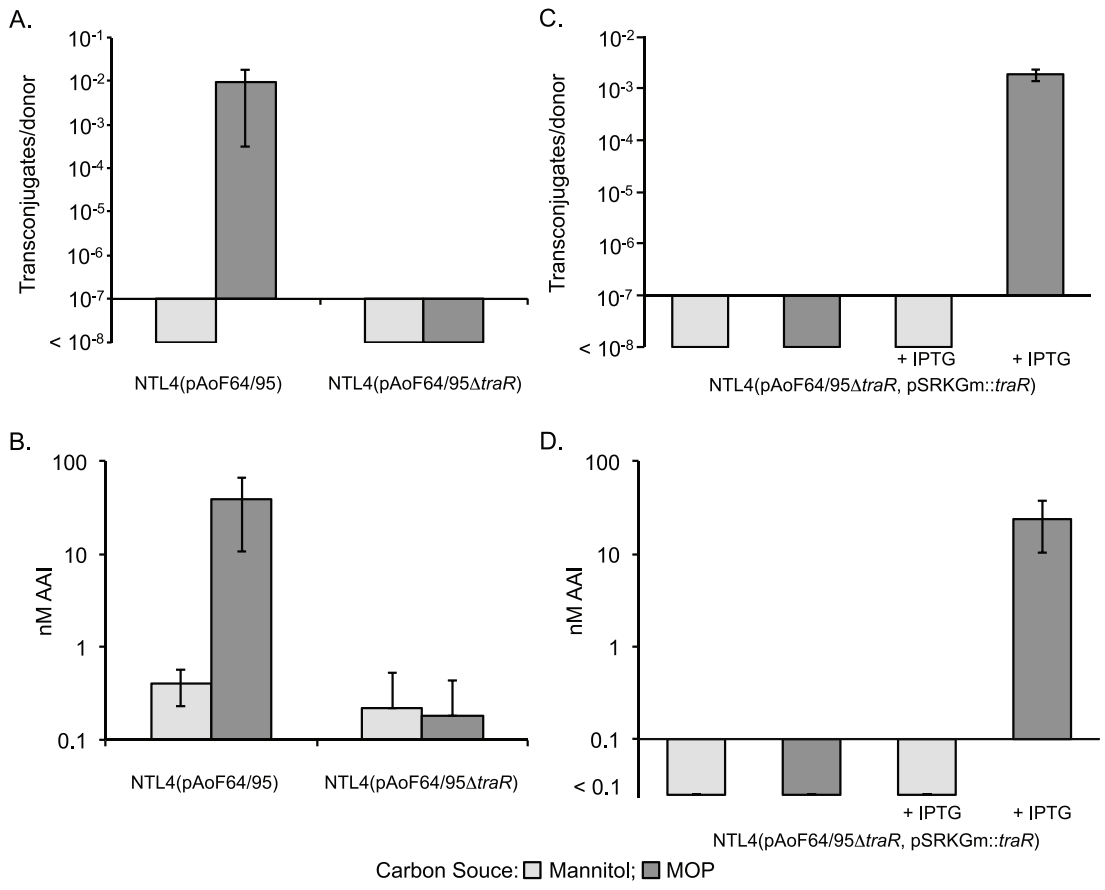


Figure 2.11 TraR is essential for induction of acyl-HSL production and conjugative transfer. A and **B.** NTL4(pAoF64/95) and the *traR* mutant, NTL4(pAoF64/95Δ*traR*), were grown in AB medium containing mannitol or MOP as primary carbon sources. After overnight growth, samples were removed and tested 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.

IPTG, conjugative transfer and acyl-HSL production were restored to approximately wild-type frequencies (Figure 2.11 C 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 (Figure 2.11 C and D).

2.5.7 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 (116, 152). 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 anti-activator, the *traM* mutant produced high levels of AAI and transferred the plasmid at high frequency, even when grown with mannitol, a noninducing substrate (Figure 2.12 A 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, that 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 (220). To test this, we passaged the complemented mutant in AB minimal media 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 (Figure 2.12 C 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 cells were grown with MOP (Figure 2.12 C and D). We reasoned that overexpressing TraM should keep conjugative transfer repressed, even in cells grown with the inducing opine (Figure 2.12 C).

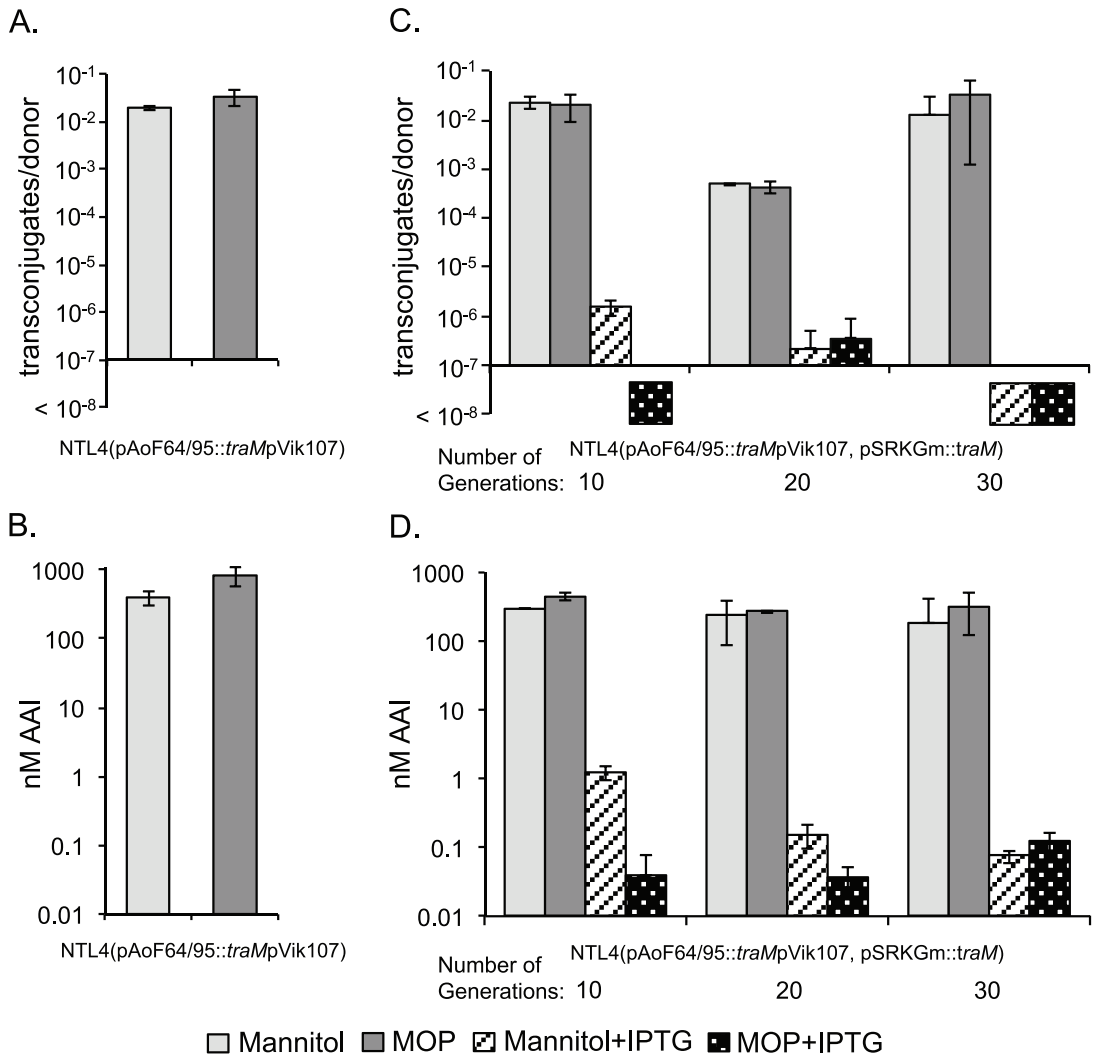


Figure 2.12 TraM inhibits TraR-dependent induction of AAI production and conjugative transfer. The *traM* mutant, NTL4(pAoF64/95::*traM*pVik107), was assessed for conjugative transfer (**A**) and AAI accumulation (**B**) when grown on AB medium supplemented with either mannitol or MOP. The mutant was 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 section 2.4.16. 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 section 2.4.7. Numbers of generations indicated at the bottom of C and D are approximate. Each experiment was done two or three times and the mean and standard deviation are shown.

2.6 Discussion

2.6.1 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 2.2). In all five cases the transmissible trait is associated with a large conjugative plasmid and, as judged by restriction enzyme analysis (Figure 2.2), these five MOP-inducible plasmids are closely related. All five of these isolates are biovar 2 strains, but only two are demonstrably pathogenic (Table 2.2). 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 2.2).

2.6.2 pAoF64/95 is an opine catabolic plasmid that encodes 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, indicates that these elements are not Ti plasmids; pAoF64/95 lacks a T-region and the genes of the *vir* regulon (Figure 2.5). Instead, it is an opine-catabolic plasmid and encodes 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 (Figure 2.7) (132) while the *moc* locus confers utilization of MOP, MOA and AGA (Figures 2.1 and 2.6). Strain F64/95 does not utilize agropine, the fourth member of the

mannityl opine family (Figure 2.1). Consistent with this observation, pAoF64/95 lacks *agcA*, which codes for the enzyme that converts agropine to MOP (54, 108, 110), and also the *agt* operon that encodes the agropine transporter (109, 110). While the overall organization of the *moc* locus is similar to that of the octopine-type Ti plasmids, there are some rearrangements (Figure 2.6). 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 (Figure 2.6). pAoF64/95 encodes an additional novel gene in the mannopine catabolism region, *mrtR*, which codes for a product that aligns to 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 (Figure 2.5 and 2.8). 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 the *motABCDtrlR* 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.

2.6.3 pAoF64/95 encodes a conserved conjugative transfer system that is most similar in gene organization to Ri and *Rhizobium* plasmids

The 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 [(178) and reviewed in (72)]. The *trb* operon, which codes for the Type IV mating bridge, as well as TraI, the acyl-HSL synthase, is invariably directly linked and divergently oriented to *repA* of the *repABC* operon [reviewed in (72)]. Moreover, in Ti plasmids, the *tra* complex and the *trb-rep* locus are separated by 60-85 kb of sequence.

The organization of these loci in pAoF64/95 more closely resembles that of megaplasmids from *A. rhizogenes* isolates including pRi1724, pRiA4 (Figure 2.8) and pRi2659, as well as some *Rhizobium* plasmids including pRL1JI (44) and p42a (243). 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 (Figure 2.8).

2.6.4 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 (Figure 2.11). However, complementation analysis showed some differences in comparison to 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 (82, 177, 189). 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 (Figure 2.11). 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.

2.6.5 Activation 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 (116, 152); mutants lacking *traM* transfer constitutively but at frequencies lower than those observed in wild-type strains induced by growth with their conjugative opine (81, 114). 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 (81, 114).

pAoF64/95 also encodes a *traM* gene and a *traM* mutant of this plasmid is constitutive for transfer (Figure 2.12). When this mutant is complemented by overexpression of *traM*, transfer is abolished, even when the cells are grown with mannitol (Figure 2.12). 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 (Figure 2.12). This observation coupled with the data concerning complementation of the *traR* mutant (Figure 2.11) discussed above, suggest 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.

2.6.6 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 hairy roots of naturally infected rose plants (167) in California (14), has three well-studied plasmids: pRiA4, pArA4, and a cointegrate of the two (255). 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 (Figure 2.2). Consistent with this physical relatedness, like pAoF64/95 this plasmid codes for catabolism of MOP, MOA and AGA, but not agropine (185) as well as utilization of agrocinopines (103).

These related plasmids all are found in independent isolates of *Agrobacterium* spp. While these plasmids are not virulence elements, based on the pathogenicity properties of the host isolates (Table 2.2) 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 encodes the genes for synthesis by the plant neoplasia of all four mannityl opines and the agrocinopines (182, 214). pRiA4, however, only confers utilization of AGR while pArA4 codes for the uptake and catabolism of AGA, MOA, MOP and the agrocininopines (185). 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 these bacteria to utilize opines produced by neoplasias induced by a virulent strain of *Agrobacterium*. Such opine-utilizing, nonpathogenic isolates of *Agrobacterium* repeatedly have been cultured from pathogen-induced neoplasias (3, 126, 127, 169, 175). 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.

2.6.7 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 (Figure 2.5, lavender colored genes). This region, about 94 kb in size, includes ORFs that are annotated as putative unknown opine transport and metabolic genes (158, 171). Likewise, the region contains sequences that align to sugar transporters and glycerol metabolism genes (158, 171), 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 (Figure 2.5, genes in green around 140 kb on the map). 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 (136, 210). The parental strain of pRi2659, also a biovar 1 strain, was isolated in the UK from a similarly diseased cucumber plant (158). Based on sequence analysis, pRi1724 and pRi2659 are closely related plasmids (171). Strain F64/95, on the other hand, is a nonpathogenic biovar 2 isolate that was cultured from an apple crown gall in California (Table 2.2). 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 evolve by recombination with other rhizobial plasmids (22, 79, 92, 171, 219).

2.6.8 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 expression of some other, to date 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.

Chapter 3: The *repABC* Plasmids with Quorum-Regulated Transfer Systems in Members of the Rhizobiales Divide into Two Structurally and Separately Evolving Groups

3.1 Notes and acknowledgments

This chapter was adapted from a paper published in *Genome Biology and Evolution* entitled "The *repABC* plasmids with quorum-regulated transfer systems in members of the Rhizobiales divide into two structurally and separately evolving groups," December 2015, Volume 7, Pages 3337-3357, with authors Margaret E. Wetzel, Gary J. Olsen, Vandana Chakravartty, and Stephen K. Farrand. Gary J. Olsen conferred with the authors on phylogenetics and wrote a program that resulted in the heat map shown in Figure 3.8. Vandana Chakravartty constructed the $traR_{pRi1724}$ expression vector and the cloned *oriT* and *traA* promoter reporter system for pRi1724. Vandana did the initial testing of $TraR_{pRi1724}$ activation of the $traA_{pRi1724}$ promoter fused to *lacZ*, which I later repeated. The authors would also like to thank Clay Fuqua of Indiana University for providing them with the strain containing the *traM* mutant of pTiR10 and Rachel J. Whitaker for her input on this chapter.

3.2 Summary

The large *repABC* plasmids of the order Rhizobiales with Class I quorum-regulated conjugative transfer systems often define the nature of the bacterium that harbors them. These otherwise diverse plasmids contain a core of highly conserved genes for replication and conjugation raising the question of their evolutionary relationships. In an analysis of 18 such plasmids these elements fall into two organizational classes, Group I and Group II, based on the sites at which cargo DNA is located. Cladograms constructed from proteins of the transfer and quorum-sensing components indicated that those of the Group I plasmids, while coevolving, have diverged from those coevolving proteins of the Group II plasmids. Moreover, within these groups the phylogenies of the proteins usually occupy similar, if not

identical, tree topologies. Remarkably, such relationships were not seen among proteins of the replication system; while RepA and RepB coevolve, RepC does not. Nor do the replication proteins coevolve with the proteins of the transfer and quorum-sensing systems. Functional analysis was mostly consistent with phylogenies. TraR activated promoters from plasmids within its group, but not between groups and dimerized with TraR proteins from within but not between groups. However, *oriT* sequences, which are highly conserved, were processed by the transfer system of plasmids regardless of group. We conclude that these plasmids diverged into two classes based on the locations at which cargo DNA is inserted, that the quorum-sensing and transfer functions are coevolving within but not between the two groups, and that this divergent evolution extends to function.

3.3 Introduction

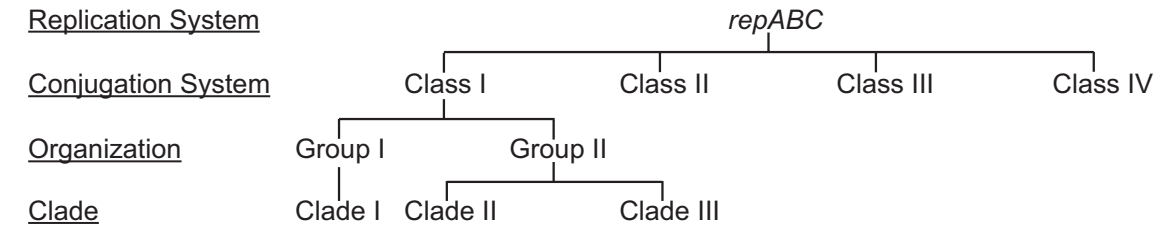
Most of the large plasmids native to members of the Rhizobiales share in common a *repABC*-type replication system (21, 25, 188). This system is remarkable in that it can acquire, stably maintain, and vertically transfer large amounts of genetic information thereby conferring a large number and wide variety of functions to its host. The best described of these plasmids carry genes that are involved in plant-microbe interactions, the functions of which often confer the defining characteristic to the host bacterium. For example the Ti (tumor inducing) and Ri (root inducing) plasmids of *Agrobacterium* spp. encode most of the virulence genes responsible for plant diseases correspondingly called crown gall and hairy root [reviewed in (232)]. In addition, Ti and Ri plasmids, as well as the Ao (*Agrobacterium* opine catabolic) and accessory plasmids of pathogenic and nonpathogenic isolates of *Agrobacterium* spp., may encode genes for ancillary traits including uptake and catabolism of opines, unique organic conjugates that are produced by crown gall tumors and hairy roots [reviewed in (55)]. Other members of the Rhizobiales including species of *Rhizobium*, *Ensifer* and *Sinorhizobium* also harbor such *repABC* plasmids, the most recognizable of these being the Sym plasmids that confer nodulation and nitrogen

fixation when the bacteria are in symbiosis with a suitable plant host (24, 86). In some cases these large *repABC* plasmids have evolved or are evolving into second chromosomes, now called chromids (101). For example, the 1.7 Mb *repABC* element in *Sinorhizobium meliloti* and the 0.5 Mb *repABC* replicon in *Rhizobium etli* are chromids (58, 101, 139).

Many of these *repABC* family plasmids also encode a conjugative transfer system responsible for horizontal transfer of the plasmid among and between bacterial species. There are at least four classes of such transfer systems associated with the *repABC* plasmids (Figure 3.1 A) [(89) and reviewed in (59)], two of which are well-characterized. The Class I system is composed of a chimeric IncQ- and IncP-like DNA metabolism (Dtr) and *oriT* region (41, 71), the former of which is encoded by the *traAFBH* and *traCDG* operons. The mating pair formation (Mpf) system is composed of an IncP-like type IV secretion system (T4SS) (142). The Class II system is composed of an IncQ-like *oriT* region and a Dtr system similar to that of the Class I system, but lacking the *traF*, *traB* and *traH* genes (31). The Mpf system of Class II transfer systems, called *avhB,* is similar to the pathogenesis-associated VirB type IV secretion systems of the Ti and Ri plasmids and of species of *Brucella* and *Bartonella* (31). The large genetic carrying capacity and relatively broad replication host range lends an overarching level of importance of these transfer systems to the evolution of traits carried by these plasmids, as well as the host bacteria that harbor them. Classes I and II transfer systems have known regulatory mechanisms; the former being controlled by a quorum-sensing mechanism while the latter is regulated by a *rctA*/*rctB*-like mechanism [reviewed in (59)]. While the nature of the inducing signal, if any, for Class II systems remains unknown, the genes and regulation of Class I transfer systems are well-studied.

Perhaps the best-characterized Class I *repABC*-associated transfer systems are those of the Ti plasmids of *A. tumefaciens*. These plasmids encode most of the *cis*- and *trans*-acting functions required by the bacterium to induce crown gall tumors on

A.



B.

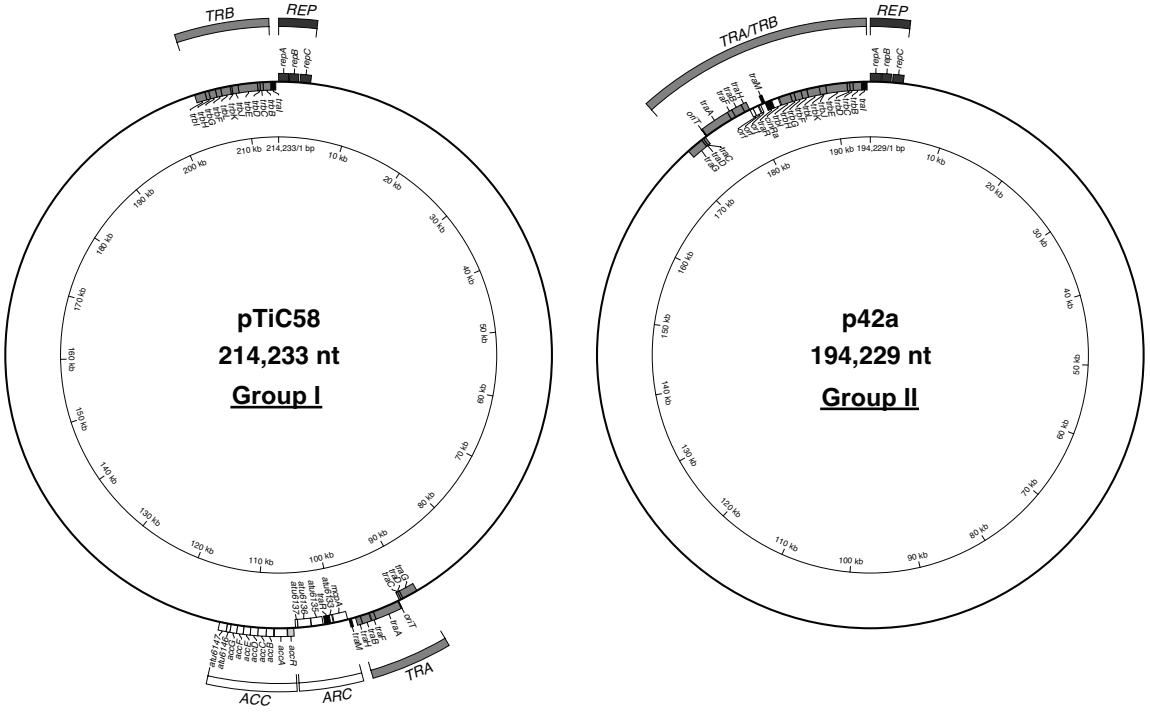


Figure 3.1 The *repABC* plasmids with Class I conjugative transfer systems in *Agrobacteria* and *Rhizobium* divide into two organizational groups. A. Flow chart categorizing *repABC* plasmids with Class I conjugative transfer systems. Plasmids with Class I transfer and regulatory genes have two distinct organizations of the genes involved in transfer and QS regulation, which can be further divided into three evolutionary clades. **B.** Examples of the two organizational Groups. Left: pTiC58 from *A. tumefaciens* strain C58, a representative of plasmids with Group I organization. Right: p42a from *R. etli* strain CFN 42, a representative of plasmids with Group II organization. The *traI*, *traR* and *traM* genes are in black, the *repABC* genes are in dark grey, and the *tra* and *trb* genes are in medium grey. On pTiC58, genes involved in regulation of transfer of pTiC58 include *accR* in light grey, and the genes for catabolism of agrocinopines A+B, the conjugative opine, in white.

susceptible host plants. It has been known for more than 40 years that conjugative transfer of the Ti plasmids is highly regulated, and is strongly inducible by one or more of the opines produced by the crown gall tumors induced by the bacterium [reviewed in (55, 72)]. Thus, induction of the transfer system of these elements is intimately linked to the habitats resulting from the pathologies induced by the bacteria.

Although opines induce transfer of Ti, and some Ao and accessory plasmids, in all studied cases transcription of the genes of the Class I transfer systems of these plasmids is directly regulated by a LuxR-family quorum-sensing (QS) system composed of the transcriptional activator TraR and an acyl-homoserine lactone (acyl-HSL) quormone (189, 264). The acyl-HSL is a population-dependent QS signal and is a product of TraI, the acyl-HSL synthase encoded by *traI*, the first gene of the plasmid *trb* operon (115, 142). The acyl-HSL, in this case N-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C8-HSL), is bound by TraR, where it promotes dimerization and stability of the activator (194, 268, 269). The dimerized form of TraR directly activates transcription of the *tra* and *trb* operons (83, 104).

One additional component, TraM, is common to these QS systems, and serves to inhibit premature activation of the *tra* regulon by basal levels of TraR when the appropriate signal is absent (44, 81, 114). TraM, an antiactivator, functions by binding to TraR, thereby inhibiting the transcription factor (152). In the Class I systems described to date, this effect is overcome and transfer is induced by an increase in the transcription of *traR* in response to some specific external signal. Such signals include opines produced by the plant neoplasias induced by pathogenic *Agrobacterium* spp. (114, 178, 191) and in the case of *R. leguminosaurum*, an orphan LuxR homolog that responds to an acyl-HSL produced by an appropriate recipient (44).

Although the *tra*, *trb*, *rep* and QS genes are conserved among the Class I group of these large plasmids in the Rhizobiales, as first noted by Moriguchi, *et. al*. (171), the organization of these genes and operons can be divided into two categories. Here we denote these categories as Group I and Group II (Figure 3.1 A). In both groups the *traI*/*trb* operon is invariably adjacent and divergently oriented to the canonical *repABC* operon. In Group I plasmids, a locus encoding *traR*, *traM*, the two divergently oriented *tra* operons, and the *cis*-acting *oriT* is separated, often by more than 60 kb, from the *traI*/*trb* genes (Figures 3.1 B, left side and Figure 3.2, left side). Moreover, *traR* generally is located in an operon the expression of which can be regulated by a specific external signal (Figure 3.1 B, left side and Figure 3.2, left side). In the Group II plasmids the *traI/trb* operon again is divergently linked to *repABC*, but the *tra* locus is contiguous to the *trb* region with *traM* and *traR* located between these two components (Figure 3.1 B, right side and Figure 3.2, right side). Moreover, unlike the Group I plasmids, *traR* appears to be monocistronic in the Group II plasmids.

That these groups of otherwise conserved genes are organized in two different patterns, raises the question of whether the component gene systems represent divergent evolutionary lineages, and if so, whether within a lineage, the genes are coevolving in a given plasmid or group of plasmids. In this study, we analyzed the evolutionary and functional relationships of select genes of these core systems encoded by 18 plasmids from members of the families Rhizobiaceae and Bradyrhizobiaceae. Here we report that, based on amino acid sequence comparisons, the QS and transfer proteins belonging to plasmids within Group I cluster together but separately from the orthologous proteins encoded by Group II plasmids. Additionally, in any given plasmid the QS and transfer proteins appear to evolve together, but separately from their adjacent Rep proteins. The *cis*-acting *oriT* sequences are highly conserved among all of the analyzed plasmids, and unlike the proteins, they do not neatly separate into two major clades. Functionally, TraR activates *tra* box-containing promoters within, but not between the two major

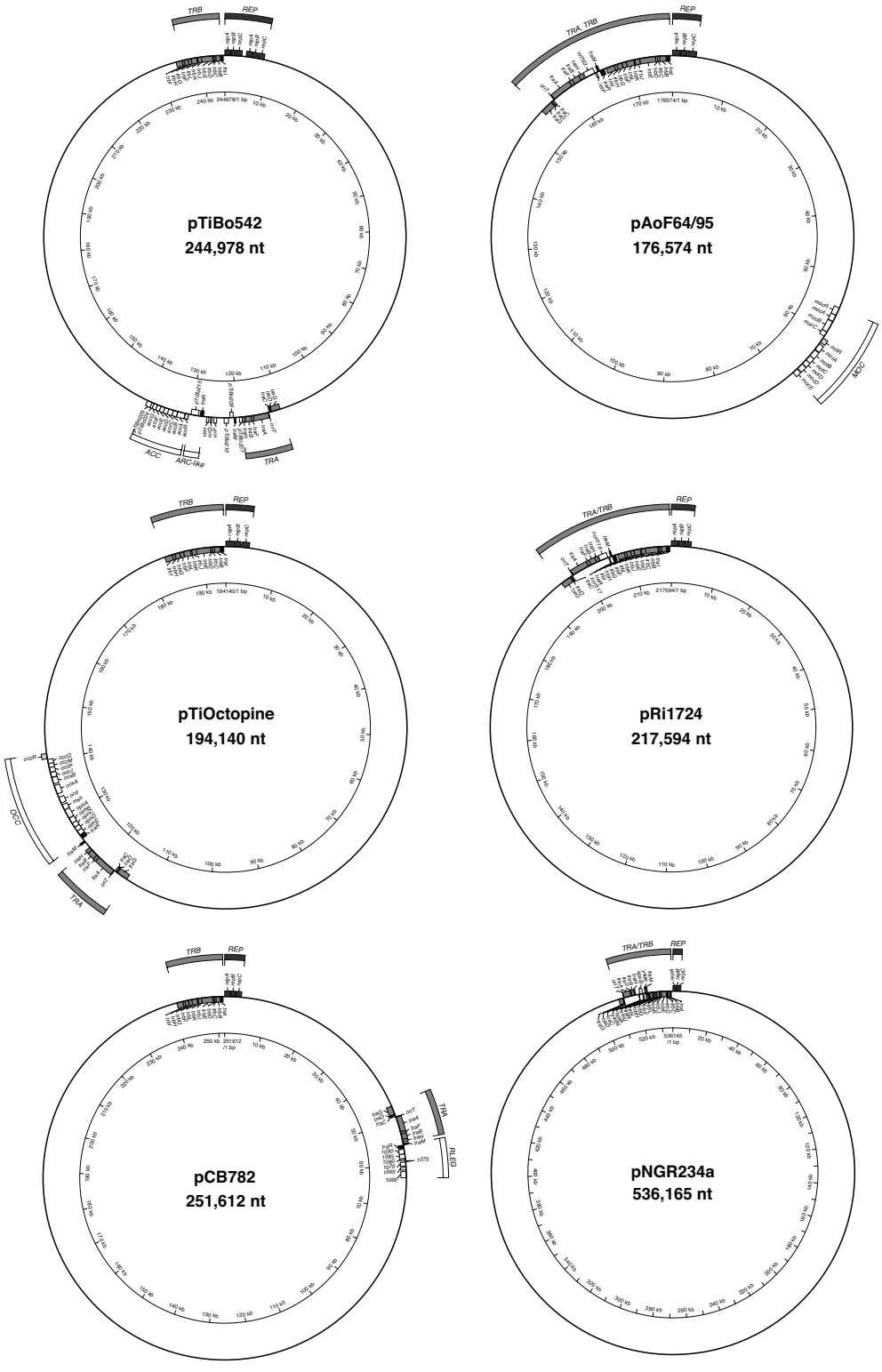

pTiBo542
244,978 nt
pAoF64/95
176,574 nt
pTiOctopine
194,140 nt
pRi1724
217,594 nt
pCB782
251,612 nt
pNGR234a
536,165 nt
Group I
Group II

Figure 3.2

Figure 3.2 (cont.) The *repABC* plasmids with Class I conjugative transfer systems in *Agrobacteria*, *Rhizobium* and *Sinorhizobium* divide into two organizational groups. On the left are three Group I plasmids; two are Ti plasmids from *A. tumefaciens* strains. The first was isolated from strain Bo542, while the second is a composite sequence from several virtually identical Octopine-type Ti plasmids. The third Group I plasmid, pCB782, is a Sym plasmid from *R. leguminosaurum bv. trifolii*. Pictured on the right are Group II plasmids from *A. radiobacter* F64/95, *A. rhizogenes* strain MAFF03 01724 and *S. fredii* strain NGR234. The *traI*, *traR* and *traM* genes are in black, the *repABC* genes are in dark grey, the *tra* and *trb* genes are in medium grey, other regulators, *accR*, *occR* and *mrtR* that are involved in regulating conjugative transfer are pictured in light grey and the catabolism region for the conjugative opine, where known or relevant, is in white.

clades. On the other hand, recombinant plasmids containing different *oriT* regions can be mobilized by plasmids from either group, but the efficiency of transfer is dependent upon how related the *oriT* is to the *oriT* that is cognate to the *trans*-acting *tra* system.

3.4 Materials and methods

3.4.1 Strains, media, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1. Cultures of *Agrobacterium tumefaciens* strain NTL4 (150) and its derivatives were grown with shaking at 30°C in liquid MG/L (20) or in AB minimal medium (20) supplemented to 0.2% with mannitol as the sole source of carbon (ABM) and 0.005% yeast extract or on solid 2% agar medium using either Nutrient both (Difco) or ABM media at 28°C. Strains of *Escherichia coli* were grown in L broth (Fischer Scientific) at 30°C or 37°C. When required for selection, antibiotics were added at the following concentrations (μg/ml): ampicillin, 100; carbenecillin, 50 or 100; gentamicin, 25; kanamycin, 25 or 50; rifampicin, 50; spectinomycin, 50 or 100; streptomycin, 50 or 100; tetracycline, 5 or 10. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a

Table 3.1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
***Agrobacterium* sp.**		
NTL4	Ti-plasmidless derivative of C58; pAtC58 Δ*tetAR*	(150)
C58C1RS	Ti-plasmidless derivative of C58; pAtC58 Rif^r Sm^r	OC
Escherichia coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k^-, m_k^-) *supE44 thi-1 gyrA relA1*	Invitrogen
Plasmids		
pKD4	Frt flanked Kan^r cassette; Amp^r	(46)
pKD46	λ Red helper plasmid; Amp^r	(46)
pMWS112	Cosmid clone of pAoF64/95 containing *mrt;* Tet^r	(252)
pMWS109	Cosmid clone of pAoF64/94 containing the *tra* operons through part of the *trb* operon; Tet^r	(252)
pRG970b	Transcriptional *lacZY* and *uidA* fusion vector; Sp^r Amp^r/$Carb^r$	(245)
pRG970b::$traA_{pAoF64/95}$	*traA-traC* intergenic region of pAoF64/95 cloned into pRG970b; *traA*::*lacZ;* Sp^r Amp^r/$Carb^r$	This work
pRG970b::$traA_{pRi1724}$	*traA-traC* intergenic region of pRi1724 cloned into pRG970b, *traA*::*lacZ*; Sp^r Amp^r/$Carb^r$	This work
pZLb251	*traA-traC* intergenic region of pTiC58 cloned into pRG970b; *traA*::*lacZ*; Sp^r Amp^r/$Carb^r$	(151)
pSRKGm	pBBR1MCS derived controlled expression cloning vector; Gm^r	(131)
pSRKGm::$traR_{pAoF64/95}$	$traR_{pAoF64/95}$ cloned into pSRKGm; Gm^r	(252)
pSRKGm::$traR_{pTiC58}$	$traR_{pTiC58}$ cloned into pSRKGm; Gm^r	(131)
pZLQ	pBBR1MCS-2 derived cloning vector; Kan^r	(151)
pZLQ::$traR_{pRi1724}$	$traR_{pRi1724}$ cloned into pZLQ; Kan^r	This work
pKK38	Broad host-range cloning vector; Tet^r	(179)
pPOKKTrlRA	$trlR_{pTi15955}$ cloned into pKK38; Tet^r	(179)
pAoF64/95Δ*mrtR*	Transfer constitutive mutant of pAoF64/95; Kan^r	This work
pTiC58Δ*accR*	Transfer constitutive mutant of pTiC58	(8)
pAoF64/95Δ*traM*	Transfer constitutive mutant of pAoF64/95; Kan^r	(252)
pTiC58Δ*traM*	Transfer constitutive mutant of pTiC58; Kan^r	(114)
pTiR10Δ*traM*	Transfer constitutive mutant of pTiR10; Kan^r	(82)

[a] Rif^r, rifampicin resistance; Sm^r, streptomycin resistance; Tet^r, tetracyclin resistance; Sp^r, spectinomycin resistance; Amp^r, ampicillin resistance; $Carb^r$, carbenecillin resistance; Gm^r, gentamicin resistance; Kan^r, kanamycin resistance.
[b] OC, our collection.

concentration of 40 μg/ml, while isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 1 mM unless otherwise stated.

3.4.2 Alignments and phylogenies

The GenBank accession numbers and information concerning the parental strains for the 18 plasmids investigated in this study are presented in Table 3.2. Plasmid pTiBo542 contains two complete copies of *repABC* in tandem direct repeat. We used the protein sequences of *repA*, *repB* and *repC* of the first repeat, which is directly linked to the *traI*/*trb* operon. Plasmid pAtK84b encodes two opine-inducible copies of *traR.* The first is inducible by agrocinopines A+B ($traR_{acc}$) and is located in close proximity to *traM* and the two *tra* operons. The second copy of *traR* is located near the nopaline catabolic operon and is inducible by nopaline ($traR_{noc}$) (178). We used the sequence of *traR* that is most closely linked with the Dtr system ($traR_{acc}$). All protein and nucleotide sequences used in this study (Table 1.1) were annotated by hand. The TrbK protein sequence of pRi1724 appears to have a premature stop codon with a conserved downstream sequence of DNA. For this study, this stop codon was annotated as unknown amino acid, X, and the downstream sequence was translated and included in the protein sequence. pNGR234a is annotated as having two consecutive reading frames containing portions of TrbE. Further analysis of the nucleotide sequence suggested to us that this was due to the addition of an extra nucleotide which changed the reading frame of this protein. For the purposes of this study, we removed the extra nucleotide and reannotated $TrbE_{pNGR234a}$. Protein sequences were aligned using three programs, MAFFT (125), ClustalW (238) and Muscle (61, 62). As MAFFT gave alignments with the best likelihood scores, all sequence alignments were conducted using this program. We tested five phylogeny programs in MEGA 6.06 (225) to construct trees for each MAFFT alignment: the Neighbor-Joining (NJ) method (200) using the bootstrap test (77) with 1000 replicates, the Minimum Evolution method (198) using 1000 replicates of the bootstrap test (77), and the UPGMA method (216). These three programs used the

Table 3.2 Descriptions and Genbank accession numbers for plasmids or contigs used in this study[a]

Genus/species	Strain(s)	Plasmid[b]	Type[c]	Group[d]	Genbank number
Agrobacterium radiobacter	F64/95	pAoF64/95*	OC	II	JX683454.1
Agrobacterium radiobacter	K84	pAtK84b*	OC	I	CP000630.1
Agrobacterium rhizogenes	A4	pRiA4b (replication)	VR	II	X04833.1
Agrobacterium rhizogenes	A4	pRiA4b (transfer)	VR	II	AB050904.1
Agrobacterium rhizogenes	K599	pRi2659	VR	II	EU186381.1
Agrobacterium rhizogenes	MAFF03-01724	pRi1724	VR	II	AP002086.1
Agrobacterium tumefaciens	Bo542	pTiBo542*	VT	I	DQ058764.1
Agrobacterium tumefaciens	Composite	pTiOctopine*	VT	I	AF242881.1[e]
Agrobacterium tumefaciens	C58	pTiC58*	VT	I	AE007871.2
Agrobacterium tumefaciens	MAFF 301001	pTi-SAKURA	VT	I	AB016260.1
Agrobacterium vitis	S4	pTiS4*	VT	I	CP000637.1
Agrobacterium vitis	S4	pAtS4c	TC	I	CP000636.1
Ensifer adhaerens	OV14	pOV14c	CR	I	CP007238.1
Nitrobacter hamburgensis	X14	pB11	CR	II	CP000322.1
Oligotropha carboxidovorans	OM5	pHCG3	CC	II	CP002827.1
Rhizobium etli	CFN 42	p42a*	CR	II	CP000134.1
Rhizobium leguminosaurum bv. trifolii	CB782	pCB782	SM	I	CP007070.1
Sinorhizobium fredii	GR64	pSfr64a*	CR	II	CP002245.1
Sinorhizobium fredii	NGR234	pNGR234a	SM	II	U00090.2

[a] The plasmids were originally identified in the species and strain indicated.

[b] *: Plasmids experimentally known to be self-conjugative.

[c] Abbreviations are: CC, carbon monoxide utilization; CR, cryptic; OC, opine catabolism; SM, symbiosis; TC, tartrate utilization; VR, virulence-rhizogenic; VT, virulence-tumorigenic.

[d] Based on the organization of the *rep*, *tra* and *trb* genes as described in the text.

[e] The sequence of the octopine-type Ti plasmid is an assembly of sequences from several virtually identical Ti plasmids including pTiR10, pTi15955, pTiA6NC, pTiAch5 and pTiB6S3 (Zhu, et al. 2000).

Poisson (270) and γ correction implemented in MEGA 6.06. The Maximum Likelihood method using a Poisson correction (270) and the Maximum Parsimony method, which uses the Subtree-Pruning-Regrafting algorithm (174) also were assessed using MEGA 6.06. The majority of the trees were either identical or highly similar and so only the trees constructed using the Neighbor Joining (NJ) method are shown.

Nucleic acid sequences were aligned using MAFFT and are visualized using the TEXshade (11) program in the SDSC biology workbench (http://workbench.sdsc.edu, last accessed December 1, 2015) alignment suite (221). The *tra* box and *oriT* trees were constructed using the NJ method with 1000 bootstrap replicates implemented in MEGA 6.06 (77, 200, 225).

3.4.3 Cloning

All polymerase chain reactions (PCR) preformed for cloning purposes used either Pfu DNA polymerase (Promega) or Phusion DNA polymerase (NEB). *traR*$_{pTiC58}$ and *traR*$_{pAoF64/95}$ were cloned into the pBBR1MCS derivative pSRKGm as previously described (131, 252). *traR*$_{pRi1724}$ was amplified by PCR using the following primers: traRpRi1724-F (5'-GCCGAATTCATATGGACGGTGACTTTCGTTCT-3') and traRpRi1724-R (5'-CGCAAGCTTTCAAACCAAGCCGTGATCTTTAGCG-3'). We directionally cloned the PCR product into pBBR1MCS derived vector pZLQ (151) using the NdeI and HindIII sites underlined in the primer sequences. To construct the *traA::lacZ* fusions and the *oriT* mobilization vectors, the *traA-C* intergenic region containing *tra* box I and the *oriT* sequence from each plasmid tested was amplified by PCR. The *traA-C* intergenic region of pAoF64/95 was amplified using primers traCpAoF64XmaI (5'-CAGATAACCCGGGATCGTCTCCTGGGTGAGAAAG-3') and traApAoF64BamHI (5'-CGAGTCCGGATCCGGTTGCGAACAATATCAAAGGG-3'), while the *traA-C* intergenic region from pRi1724 was amplified using the primers traCpRi1724XmaI (5'-CGCCCCGGGTCCGTCTGTCTCCTTGGGTG-3') and traApRi1724BamHI (5'-CGCGGATCCGGTTGCAAACGAAATCAATG-3'). These products were subsequently directionally cloned into pRG970b (245) using XmaI and BamHI (underlined in the primer sequence), such that the *traA* promoter was transcriptionally fused to *lacZ*. pZLb251 which contains the *traA-C* intergenic region from pTiC58 cloned into pRG970b is described elsewhere (151).

3.4.4 Construction of mutant strains

All PCR reactions used to construct mutant strains were carried out with either Pfu DNA polymerase (Promega) or Taq DNA polymerase (NEB). In-frame deletion mutants of *traM* and *mrtR* on pAoF64/95 were constructed using the method of Datsanko and Wanner (46). Briefly, the kanamycin resistance cassette of pKD4 was amplified using the following primers which contained 5' overhang sequences for *traM*: Forward primer, 5'-CTTGAGCGTGGGGTTTTCGAAAAAAGGGAGGAGAATGGTGT GTAGGCTGGAGCTGCTTCG-3', Reverse primer, 5'-CCTCGTCGCGATCGCCAAGGACCAC GGCCTGCTGTAGCGCATATGAATATCCTCCTTAGT-3'. The PCR product was transformed into *Escherichia coli* (pKD46) carrying a cosmid clone of the appropriate region of pAoF64/95 (252). λ red-mediated replacement of *traM* with the kanamycin cassette was confirmed in the cosmid by PCR analysis using the traMcheckdown (5'-CTATGATGTTGACGTTTGCATCTT-3') and traMcheckup (5'-GATCGCCATGACCTCTTTGA-3') primers. The mutant allele of *traM* was marker exchanged into pAoF64/95 in strain NTL4 as described previously (252). The same method was used to construct the indel mutation in *mrtR*. The kanamycin cassette of pKD4 was amplified with the following primers: forward, 5'-TTGGACACCGAGCCA ATGTACATCCAACTGCAGCAAGATGTGTAGGCTGGAGCTGCTTCG-3', and reverse, 5'-CTAGCCACCTTTGTGTGGTGTCCTACCGCCCATCATCATCATATGAATATCCTCCTTAGT-3'. The λ red-mediated *mrtR* mutation in the cosmid was confirmed by PCR using the mrtRcheckup primer (5'-GCCCTCCGCTCCCAGTTAAA-3') and the mrtRcheckdwn primer, (5'-AGCGGCTACAATCTTCCTTG-3'), and the mutant allele of *mrtR* was marker exchanged into pAoF64/95 as described previously (252).

3.4.5 β-galactosidase assays

The $traA_{pTiC58}$::*lacZ*, $traA_{pAoF64/95}$::*lacZ* and $traA_{pRi1724}$::*lacZ* reporter vectors described above were electroporated into *A. tumefaciens* strain NTL4. Strain NTL4 harboring the empty vector pRG970b (245) also was constructed as a control. We

subsequently electroporated pSRKGm, pSRKGm::*traR*$_{pTiC58}$, pSRKGm::*traR*$_{pAoF64/95}$, or pZLQ::*traR*$_{pRi1724}$ individually into each of the four *traA*::*lacZ* reporter strains. The resulting 16 strains were assessed for β-galactosidase activity on solid medium. Briefly, single colonies of each strain were individually suspended in 250 µl of 0.9% NaCl and 5 µl volumes of each suspension were spotted onto ABM media containing X-gal, spectinomycin and IPTG to induce expression of TraR. Strains were tested on medium both with and without 50 nM N-(3-oxooctanoyl)-L-homoserine lactone, *Agrobacterium* autoinducer (AAI, Sigma-Aldrich). β-galactosidase activity was assessed visually after 48 hours of incubation at 28°C.

3.4.6 Mobilization experiments

Empty vector pRG970b (245), or pRG970b containing the *traA-C/oriT* region from pAoF64/95, pTiC58 or pRi1724 was electroporated into strains NTL4(pTiC58Δ*accR*) (66) and NTL4(pAoF64/95Δ*mrtR*) (unpublished data, see above). The *repABC* plasmids in these strains are constitutive for conjugative transfer (tra^{C}). The resulting tra^{C} donor strains carrying the *oriT* vectors and the recipient strain, C58C1RS, were grown in liquid MG/L medium overnight. Filter matings were conducted as described previously (74). Briefly, donor and recipient strains were mixed together in a 10:1 volume/volume ratio and cells in 50 µl volumes of each mixture were collected by vacuum filtration onto a sterile 0.22 µm filter disc. The filter was then placed bacterial side up onto solid media and incubated at 28°C for 22-24 hours. The cells were collected by vortexing each disk in a one milliliter volume of 0.9% NaCl. Volumes of 100 µl of a decade dilution series of each mating then were plated onto solid medium supplemented with rifampicin, streptomycin, carbenecillin and spectinomycin. Rifampicin and streptomycin select for the recipient strain, while carbenecillin and spectinomycin select for the mobilized *oriT* plasmid. Colonies of transconjugates were enumerated after 5 to 6 days of incubation at 28°C. Frequencies of transfer are expressed as transconjugates per input donor (74).

3.4.7 TrlR-TraR cross-dimerization

To assess the ability of TraR to cross-dimerize with orthologous proteins, we utilized pPOKKTrlRA (179), a vector expressing a cloned copy of *trlR* from pTi15955, or pKK38 as an empty vector control. These plasmids were electroporated into *A. tumefaciens* strain NTL4 harboring the tra^{C} *traM* deletion derivatives of pTiC58 (114), pTiR10 (81) and pAoF64/95, all of which confer resistance to kanamycin. If TrlR cross-dimerizes with the TraR cognate to the system, then transfer frequencies of these tra^{C} plasmids should decrease. The resulting strains were assessed for conjugative transfer frequency using the drop-plate mating technique described previously (74, 252). Transconjugates were selected on medium containing rifampicin, streptomycin and kanamycin. Transfer frequencies are expressed as the number of transconjugates obtained per input donor.

3.4.8 Analysis of protein coevolution

Analysis of trees constructed from the RepA, RepB, RepC, TrbE, TrbK, TraI, TraR, TraM, TraG and TraA protein alignments compared to the MAFFT alignments of each protein was accomplished by using the likelihood scores. We used the formula:

(LnLikelihood(Data | GivenTree) - LnLikelihood(Data | RandomTree)) / (LnLikelihood(Data | OwnTree) - LnLikelihood(Data | RandomTree))

This formula essentially compares the fit of the sequence data in an alignment to the trees constructed from different protein alignments, with each adjusted for the component of the score attributable to random similarity of trees. Proteins that are coevolving have a score close to 1.00, while proteins that are not coevolving have scores closer to 0.00 (negative values are possible because a tree can be worse than random for the given data). The data were entered into a program written to

display output values on a grey scale heat map, with values closer to 1.00 being white and values closer to 0.00 being black.

We noticed that using relatedness values from proteins from plasmids that are highly similar skewed the entire data set toward coevolution since proteins from those plasmids will always group closely together. We therefore excluded results from analysis of pTi-SAKURA, which is highly similar to pTiC58 (94, 222), and pRi1724, which is highly similar to pRi2659 (171).

3.5 Results

3.5.1 The two distinct organizations of the genes involved in conjugative transfer and its regulation correspond to plasmid type

Structurally, all 18 of the Class I-type *repABC* plasmids studied divide into two distinct genetic organizations, Group I and Group II (see Figure 3.1 B, Table 3.2 and Figure 3.2). Common to all of the plasmids examined, the *traI*/*trb* operon is invariably adjacent to the divergently oriented *repABC* operon. However, in the Group I plasmids such as pTiC58, pTiBo542, pTiOctopine and pCB782 (Figure 3.1 B, left side, Figure 3.2, left side and Table 3.2), as well as in at least one opine catabolic plasmid (pAtK84, Table 3.2) the two *tra* operons, along with *traR* and *traM,* are located near the region encoding catabolism of the conjugative opine and are separated by a large but variable distance from the *traI*/*trb* region. In the Group II plasmids, such as the auxiliary plasmid p42a of *Rhizobium etli* (Figure 3.1 B, right side and Table 3.2), pAoF64/95, pRi1724, pNGR234a (Figure 3.2, right side and Table 3.2), two sinorhizobial plasmids, pSfr64a and pNGR234a, and the two bradyrhizobial plasmids, pHCG3 and pB11 (Table 3.2), the *tra* and *trb* regions are contiguous and *traR* and *traM* are located between the *traI*/*trb* operon and the last gene of the *traAFBH* operon. Moreover, unlike in the Group I plasmids where *traR* generally is a member of an operon, in the Group II plasmids examined to date *traR*

is monocistronic (Figure 3.1 B, right side and Figure 3.2, right side). Remarkably, all of the Ti plasmids characterized to date fall into Group I, while all of the characterized Ri plasmids fall into Group II (Table 3.2). Two of the Class I-type plasmids from species of *Rhizobium*, *Ensifer* and *Sinorhizobium* (pOV14c and pCB782) fall into Group I, while three (p42a, pSfr64a and pNGR234a) fall into Group II (Table 3.2). The two bradyrhizobial plasmids (pHCG3 and pB11) fall into Group II (Table 3.2).

3.5.2 Proteins of the quorum-sensing systems of plasmids from members of the Rhizobiales form two major clades that correspond to plasmid organization

The observation that *traR* is organized either as a member of an operon, as seen in the Group I plasmids, or is monocistronic, as in Group II plasmids, along with the bimodal organization of the *tra-trb* regulon (Figure 3.1 B and Figure 3.2), led us to hypothesize that TraR proteins that regulate conjugative transfer could be divergently evolving between the two groups of plasmids. In addition, considering that TraM interacts with TraR, and that the acyl-HSL produced by TraI is the ligand of TraR, we postulated that these three QS proteins would evolve together. To test these two hypotheses, we assessed amino acid sequence relatedness of the TraR, TraM, and TraI proteins derived from the Class I plasmids described in Table 3.2.

Figure 3.3 A-C shows the phylogenetic trees of the three QS proteins for the 18 plasmids examined, all determined as described in materials and methods. The trees built from these three proteins overall are topologically consistent. Sixteen of the protein sets divide into two major clades while two, those from the plasmids of *Oligotropha carboxidovorans* and *Nitrobacter hamburgensis*, divide as distant relatives to both of the two major clades. Whether either of these plasmids is conjugative remains to be determined. The members of the two major clades divide precisely by plasmid organization. The three proteins from Group I plasmids, in which *traR* is polycistronic and where the *tra* and *trb* regions are physically

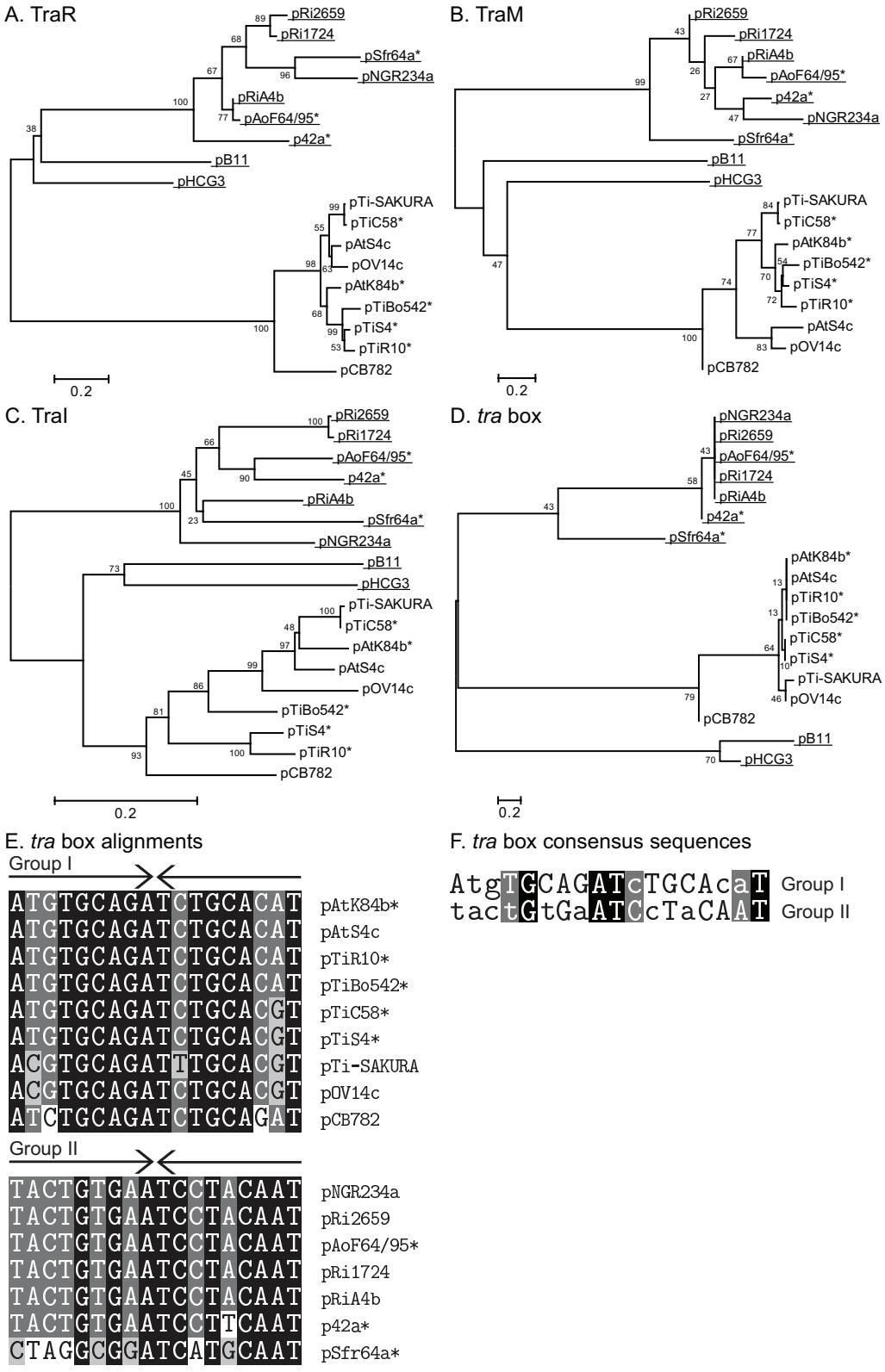

A. TraR
pRi2659
pRi1724
pSfr64a*
pNGR234a
pRiA4b
pAoF64/95*
p42a*
pB11
pHCG3
pTi-SAKURA
pTiC58*
pAtS4c
pOV14c
pAtK84b*
pTiBo542*
pTiS4*
pTiR10*
pCB782
0.2
B. TraM
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pNGR234a
pSfr64a*
pB11
pHCG3
pTi-SAKURA
pTiC58*
pAtK84b*
pTiBo542*
pTiS4*
pTiR10*
pAtS4c
pOV14c
pCB782
0.2
C. TraI
pRi2659
pRi1724
pAoF64/95*
p42a*
pRiA4b
pSfr64a*
pNGR234a
pB11
pHCG3
pTi-SAKURA
pTiC58*
pAtK84b*
pAtS4c
pOV14c
pTiBo542*
pTiS4*
pTiR10*
pCB782
0.2
D. tra box
pNGR234a
pRi2659
pAoF64/95*
pRi1724
pRiA4b
p42a*
pSfr64a*
pAtK84b*
pAtS4c
pTiR10*
pTiBo542*
pTiC58*
pTiS4*
pTi-SAKURA
pOV14c
pCB782
pB11
pHCG3
0.2
E. tra box alignments
Group I
ATGTGCAGATCTGCACAT pAtK84b*
ATGTGCAGATCTGCACAT pAtS4c
ATGTGCAGATCTGCACAT pTiR10*
ATGTGCAGATCTGCACAT pTiBo542*
ATGTGCAGATCTGCACGT pTiC58*
ATGTGCAGATCTGCACGT pTiS4*
ACGTGCAGATTTGCACGT pTi-SAKURA
ACGTGCAGATCTGCACGT pOV14c
ATCTGCAGATCTGCAGAT pCB782
Group II
TACTGTGAATCCTACAAT pNGR234a
TACTGTGAATCCTACAAT pRi2659
TACTGTGAATCCTACAAT pAoF64/95*
TACTGTGAATCCTACAAT pRi1724
TACTGTGAATCCTACAAT pRiA4b
TACTGTGAATCCTTCAAT p42a*
CTAGGCGGATCATGCAAT pSfr64a*
F. tra box consensus sequences
AtgTGCAGATcTGCAcaT Group I
tactGtGaATCcTaCAAT Group II

Figure 3.3

Figure 3.3 (cont.) TraR, TraI and TraM, and the *tra* box I sequences divide into three clades. Neighbor-Joining trees constructed from MAFFT alignments for **A.** TraR, **B.** TraM and **C.** TraI proteins and **D.** *tra* box I nucleotide sequences from 18 plasmids in the *Rhizobiales*. Bootstrap values from 1000 replicates are located next to the branches. The evolutionary distances were computed with the Poisson correction. The asterisks (*) denote plasmids that are known to be conjugative, while the underscores indicate plasmids with a Group II organization as described in the text. **E.** Alignment of *tra* box I nucleotide sequences from the 16 Group I and Group II plasmids. **F.** The consensus sequences of *tra* box I from Group I and Group II plasmids, excluding the two plasmids from Clade III, pB11 and pHCG3. Capital letters in the consensus sequences indicate invariant bases, while lower case letters are conserved nucleotides. The black color indicates invariant bases and grey indicates conserved bases.

separate on the plasmid, form one clade while the three proteins from Group II plasmids, in which *traR* is monocistronic and the *tra* and *trb* regions are adjacent to one another, group together as the second clade.

3.5.3 The *tra* box sequences, while having a small core set of nucleotides in common, divide into two distinct conserved sequences: Group I- and Group II-like

TraR binds to an inverted repeat sequence, called the *tra* box, from which it activates transcription from the promoters of the associated operons (151, 268). The Class I rhizobial and Ti plasmids typically contain between two and four such boxes (44, 81, 83, 104, 253). *tra* box I, located in the intergenic region between and controlling transcription of the divergently oriented *traAFBH* and *traCDG* operons, is the most highly conserved of these sequences [reviewed in (253)]. *tra* box II is located upstream of and controls transcription of the *traI/trb* operon, while *tra* box III, when present, is located just upstream of *tra* box II and contributes to the control of transcription of the divergently oriented *repABC* operon (143, 181). A fourth and less conserved *tra* box, *tra* box IV, was described in the Ti plasmids and is located in the promoter region of *traM* (81). We aligned the nucleotide sequences of the putative *tra* box I from each of the 18 plasmids examined in this study.

Like TraR, TraM and TraI, the putative *tra* boxes divide into two distinct sets of sequences corresponding to the Group I and Group II clades (Figure 3.3 D and E). The *tra* box I sequences from six of the Group II plasmids are virtually identical with one sequence, that from p42a, differing by a single nucleotide and that from pSfr64a differing at eight nucleotides (Figure 3.3 E). On the other hand, the sequences of the Group I clade subdivide into groups that represent changes to five separately located bases (Figure 3.3 D and E). The Group I *tra* box I consensus sequence and the Group II *tra* box I consensus sequence share in common only four fully conserved bases, while three additional bases are conserved in the *tra* box I sequences of most of the Rhizobial plasmids examined (Figure 3.3 F). Additionally, the Group I sequences form a considerably more perfect inverted repeat in comparison to the Group II sequences (Figure 3.3 E and F). Consistent with the divergence of their TraR proteins, the putative *tra* box I sequences of pB11 from *N. hamburgensis* and pHCG3 from *O. carboxidovorans* are more distantly related to the boxes of Group I and Group II plasmids, and most closely related to each other (Figure 3.3 D).

3.5.4 TraR activates transcription from a *tra* box-dependent promoter among members of the same clade, but not between members of the other clade

Given the division of TraR and its DNA binding site into two major groups, we tested the ability of activators of each of the two major clades to induce transcription from cognate and noncognate *tra* boxes. This was accomplished by assessing activation of cognate and noncognate promoters from the *traAFBH* operon by alleles of *traR* from different sources. We cloned *traR* from pTiC58 ($traR_{pTiC58}$), pAoF64/95 ($traR_{pAoF64/95}$) and pRi1724 ($traR_{pRi1724}$) into pBBR1MCS-derived vectors (131, 151), and constructed *ptraA*::*lacZ* transcriptional fusions that contain the entire *traA-traC* intergenic regions, including the *tra* box I sequences, from pTiC58 ($ptraA_{pTiC58}$), pAoF64/95 ($ptraA_{pAoF64/95}$) and pRi1724 ($ptraA_{pRi1724}$), all as described in materials and methods. The three TraR orthologs were tested for their ability to activate

transcription of the three *traAFBH* promoters as assessed by β-galactosidase activity in cultures grown with and without 3-oxo-C8-HSL (AAI).

In cells grown in the absence of the quormone, $TraR_{pTiC58}$ minimally activated only its cognate promoter (Figure 3.4 A). When AAI was added to the medium, $TraR_{pTiC58}$ strongly activated only $ptraA_{pTiC58}$ (Figure 3.4 B). No significant levels of β-galactosidase activity were detected in any growth condition from strains in which $TraR_{pTiC58}$ was paired with the *traA* promoter-reporters from pAoF64/95 or from pRi1724 (Figure 3.4 A and B). In cells grown in the absence of AAI, $TraR_{pAoF64/95}$ failed to activate expression of β-galactosidase from any of the three *ptraA* promoters (Figure 3.4 A). However, when grown with the acyl-HSL, the strain expressing $TraR_{pAoF64/95}$ strongly activated its cognate promoter and to a lesser extent $ptraA_{pRi1724}$ (Figure 3.4 B). $TraR_{pAoF64/95}$ also activated $ptraA_{pTiC58}$ in an AAI-dependent manner, but at only a barely detectible level (Figure 3.4 B). Unexpectedly, when grown with AAI, $TraR_{pRi1724}$ did not activate any of the reporters, including its cognate promoter, $ptraA_{pRi1724}$ (Figure 3.4 B). However, in strains grown without AAI, $TraR_{pRi1724}$ activated $ptraA_{pAoF64/95}$, and to a lesser extent its cognate promoter, and very minimally $ptraA_{pTiC58}$ (Figure 3.4 A).

3.5.5 TraR exhibits dimerization specificity delineated by the Group I- and Group II-like clade structure

The N-terminal region of TraR contains both a quormone -binding domain and the primary dimerization domain (248, 265). We assessed whether the proteins from different clades can cross-dimerize by using TrlR, a dominant-negative mutant of TraR encoded by the octopine-type Ti plasmids pTi15955 and pTiR10 (179, 267). TrlR has functional ligand-binding and dimerization domains, but lacks the C-terminal DNA-binding domain (28, 179, 267). When coexpressed TrlR strongly inhibits the activity of $TraR_{Octopine}$ by forming inactive heterodimers (28, 179, 267).

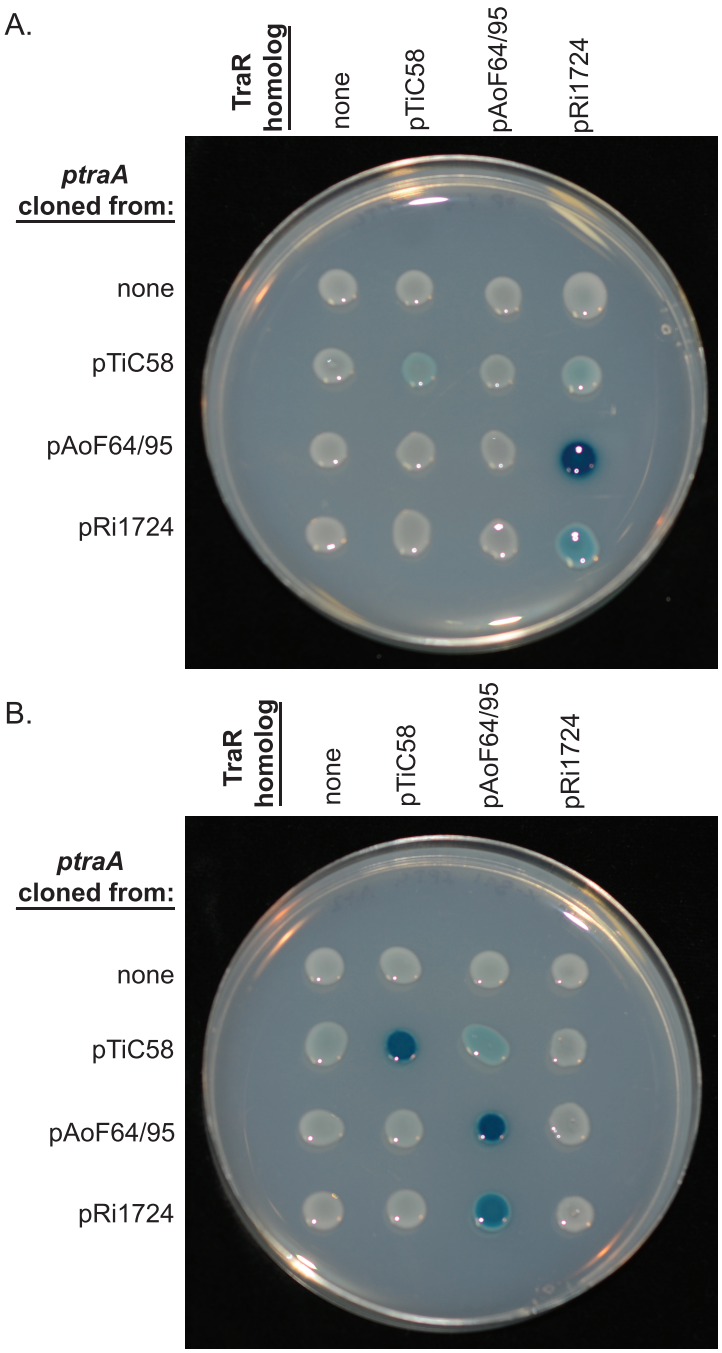


Figure 3.4 TraR activates transcription of the *traAFBH* promoters within but not between clades. Derivatives of strain NTL4 carrying a *traA::lacZ* transcriptional fusion from one of three plasmids (labeled on the vertical axis) and one of three TraR orthologs (labeled on the horizontal axis) were assessed for β-galactosidase activity on solid ABM media supplemented with X-gal and IPTG to induce expression of *traR*. The growth medium in plate B is additionally supplemented with AAI at 50 nM.

We assessed the ability of different TraR proteins to cross-dimerize indirectly by evaluating conjugative transfer frequencies of derivatives of *A. tumefaciens* strain NTL4 expressing both $TrlR_{pTi15955}$ and TraR of the native plasmid. We utilized transfer-constitutive (*tra*^{C}) strains (see Table 3.1) harboring either pTiC58Δ*traM* or pTiR10Δ*traM* as representatives of the Group I plasmids and pAoF64/95Δ*traM* as a representative of the Group II plasmids. We constructed these three strains to additionally carry a plasmid with either a cloned wild-type copy of *trlR* (pPOKK*trlR*A) or the empty vector, pKK38 (179). If transfer frequencies decrease in the *tra*^{C} strain harboring pPOKK*trlR*A in comparison to the *tra*^{C} strain harboring pKK38, then TrlR is functionally dimerizing with the native TraR thereby inhibiting activation of the *tra* regulon.

In the donor harboring pTiR10Δ*traM*, a Group I plasmid essentially identical to pTi15955, expression of TrlR inhibited the transfer frequencies of the megaplamid by about 5,500-fold in comparison to the same *tra*^{C} strain harboring the empty vector (Table 3.3). Transfer frequency of the donor harboring pTiC58Δ*traM*, another Group I plasmid, decreased by approximately 140-fold when TrlR was expressed in comparison to the same strain without TrlR (Table 3.3). In donors harboring the Group II plasmid pAoF64/95Δ*traM* expression of TrlR had only a negligible effect on the transfer frequency of the opine-catabolic plasmid when compared to the same strain without TrlR (Table 3.3).

3.5.6 Structural genes of the conjugative transfer system divide into Group I and Group II clades

To assess whether, like the quorum-sensing proteins, the proteins of the conjugative transfer systems of Group I and II plasmids have divergently evolved we included in our study sequence comparisons of the products of three essential, and one nonessential transfer genes, two from the DNA metabolism (Dtr) operons and two

Table 3.3 TrlR dimerizes with TraR from Group I, but not Group II plasmids

Tra^{C} plasmid[a]	Plasmid[b]	*trlR*	Conjugation frequency[c]	Fold inhibition[d]
pTiR10Δ*traM*	None	-	1.5×10^{-4}	NA[e]
pTiR10Δ*traM*	pKK38	-	8.2×10^{-5}	1.8
pTiR10Δ*traM*	pPOKK*trlR*A	+	1.5×10^{-8}	5,500
pTiC58Δ*traM*	pKK38	-	8.0×10^{-5}	NA
pTiC58Δ*traM*	pPOKK*trlR*A	+	5.8×10^{-7}	140
pAoF64/95Δ*traM*	pKK38	-	1.2×10^{-2}	NA
pAoF64/95Δ*traM*	pPOKK*trlR*A	+	7.7×10^{-3}	1.6

[a] All in *A. tumefaciens* strain NTL4.

[b] *trlR* was expressed from pPOKK*trlR*A (Table 3.1).

[c] Expressed as the number of transconjugates recovered per input donor cell. Each cross was performed in duplicate and the values presented are the mean of each cross.

[d] Calculated by dividing the transfer frequency of the tra^{C} strain harboring an empty vector by the transfer frequency of the donor harboring the *trlR* expressing vector, or by dividing the tra^{C} strain by the same tra^{C} strain harboring an empty vector.

[e] NA: Not applicable.

from the mating pair formation (Mpf) operon (Table 1.1). TraA, an essential representative of the Dtr system, is the strand transferase responsible for nicking the *oriT* and is a component of the relaxosome (38). The protein contains two conserved motifs, a tyrosine-type site-specific recombinase domain and a helicase domain (73). The second protein, TraG, is the coupling factor that is believed to interface the relaxosome with the T4SS (98). TraG, which is essential, is distantly related to VirD4, a component involved in plant virulence, and contains two potential nucleotide-binding domains (38, 73, 98). TrbE, an essential representative of the Mpf system, contains a Walker type-A nucleoside triphosphate binding domain and is distantly related to VirB4 of the T4SS involved in plant virulence (142, 144), whereas TrbK, which is not essential for transfer, is a short protein and is involved in entry exclusion (37). TraA, TraG and TrbE are large multi-domain proteins making them excellent representatives of the transfer system. Like those

of the quorum-sensing proteins, trees of TraA, TraG and TrbE divide into two major clades, precisely corresponding to the Group I and Group II organizational patterns (Figure 3.5 A, B and C). Each of the three essential transfer proteins from *Oligotropha* and *Nitrobacter* are related to each other, but form a distinct third clade (Figure 3.5 A, B and C). TrbK divides into two major clades that are mostly consistent with the two organizational types (Figure 3.5 D). However, two TrbK sequences, those from pTiR10 and pTiS4, always group together and although they most often form a clade with other plasmids in Group I (Figure 3.5 D), these two proteins occasionally form a separate branch depending upon which program was used to align the amino acid sequences and which program was used to construct the cladograms (data not shown). Additionally for all trees constructed with the TrbK alignments, pSfr64a always groups separately from the other Group I plasmids, and most often groups with the two bradyrhizobial plasmids, pB11 and pHCG3 (Figure 3.5 D).

3.5.7 The Dtr region of pTi-SAKURA is chimeric

Although the trees group the three QS proteins, TrbE, and TrbK of pTi-SAKURA and pTiC58 closely together (Figure 3.3 A, B, C and Figure 3.5 C and D), the cladograms constructed using TraA and TraG sequences indicate that these proteins of pTi-SAKURA are not most closely related to those of pTiC58 (Figure 3.5 A and B). Considering the incongruous cladograms for TraA and TraG and the proximal location of the two genes encoding these protein to *traM* and *traR* (Figure 3.6 A), we hypothesized that this region of the two Ti plasmids differs in its phylogenies due to a recombination event. To examine this possibility, we constructed phylogenetic trees of the protein products of the remaining *tra* genes, *traF*, *traB*, *traH*, *traC,* and *traD* (Table 1.1). In comparing these proteins from the entire group of 18 plasmids, the trees for TraB, TraH, TraC, and TraF yielded Group I- and Group II-like clades (Figure 3.6 E, F, C and D). However, the cladogram for TraD exhibited anomalies (Figure 3.6 B); the tree did yield Group II-like clades, but a few members of the

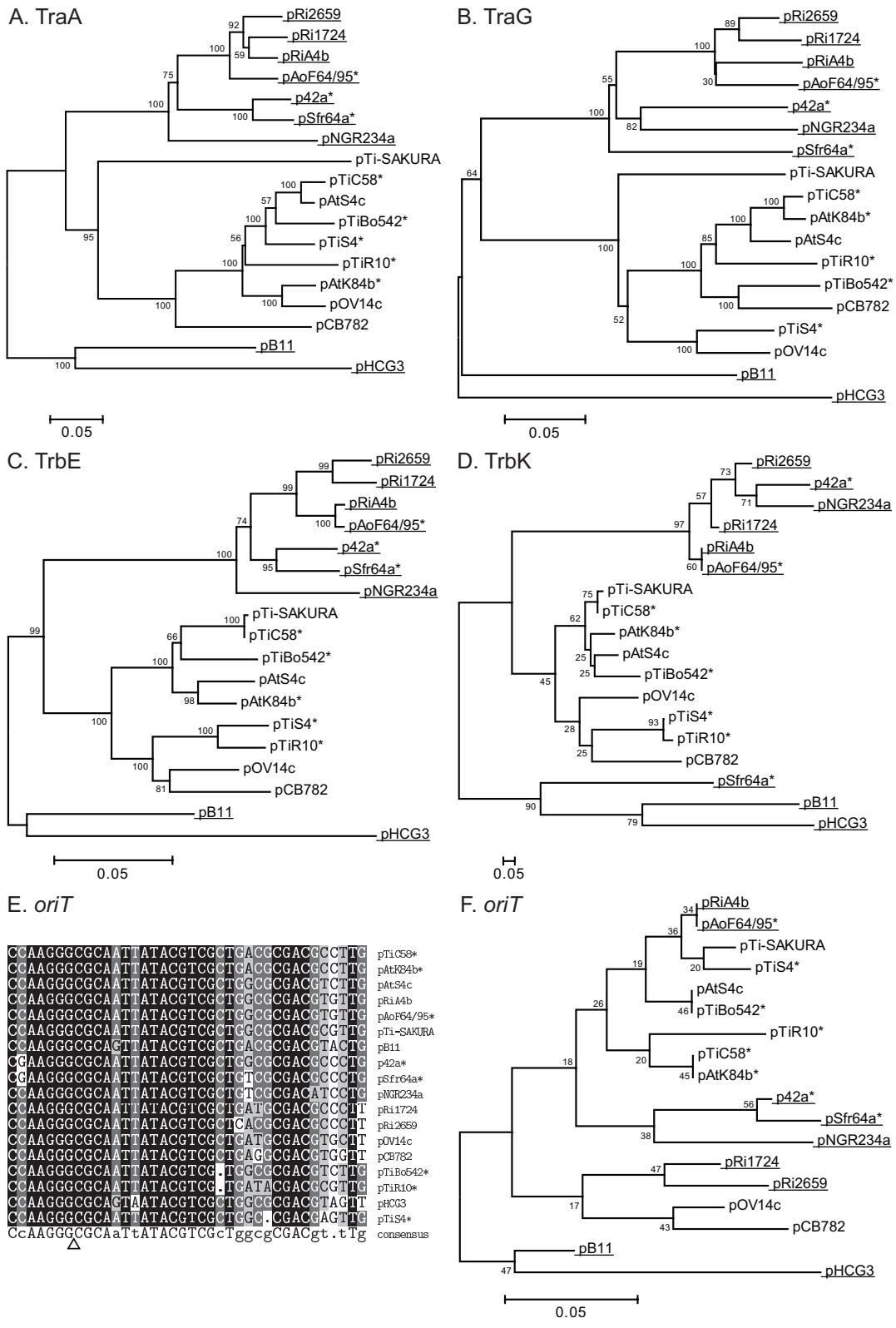

A. TraA
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pSfr64a*
pNGR234a
pTi-SAKURA
pTiC58*
pAtS4c
pTiBo542*
pTiS4*
pTiR10*
pAtK84b*
pOV14c
pCB782
pB11
pHCG3
0.05
B. TraG
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pNGR234a
pSfr64a*
pTi-SAKURA
pTiC58*
pAtK84b*
pAtS4c
pTiR10*
pTiBo542*
pCB782
pTiS4*
pOV14c
pB11
pHCG3
0.05
C. TrbE
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pSfr64a*
pNGR234a
pTi-SAKURA
pTiC58*
pTiBo542*
pAtS4c
pAtK84b*
pTiS4*
pTiR10*
pOV14c
pCB782
pB11
pHCG3
0.05
D. TrbK
pRi2659
p42a*
pNGR234a
pRi1724
pRiA4b
pAoF64/95*
pTi-SAKURA
pTiC58*
pAtK84b*
pAtS4c
pTiBo542*
pOV14c
pTiS4*
pTiR10*
pCB782
pSfr64a*
pB11
pHCG3
0.05
E. oriT
pTiC58*
pAtK84b*
pAtS4c
pRiA4b
pAoF64/95*
pTi-SAKURA
pTiBo542*
pAtS4c
pTiS4*
pTiR10*
pOV14c
pCB782
pSfr64a*
pNGR234a
pRi1724
pRi2659
pOV14c
pCB782
pB11
pHCG3
consensus
F. oriT
pRiA4b
pAoF64/95*
pTi-SAKURA
pTiS4*
pAtS4c
pTiBo542*
pTiR10*
pTiC58*
pAtK84b*
p42a*
pSfr64a*
pNGR234a
pRi1724
pRi2659
pOV14c
pCB782
pB11
pHCG3
0.05

Figure 3.5

Figure 3.5 (cont.) TraA, TraG, TrbE and TrbK divide into two major clades, whereas the *oriT* sequences do not. Neighbor-Joining trees of the individual proteins from 18 plasmids constructed from MAFFT alignments are shown. Bootstrap values from 1000 replicates are located next to the branches. The evolutionary distances were computed with the Poisson correction. **A.** TraA, **B.** TraG, **C.** TrbE and **D.** TrbK. **E.** The nucleotide sequences of the *oriT* region of the 18 selected Class I plasmids were aligned with MAFFT. The black color indicates invariant bases, while the bases colored dark grey are highly conserved. Nucleotides colored light grey and white show less conservation. Capital letters in the consensus sequences indicate invariant bases, whereas lower case letters are bases conserved in 50% or more of the sequences. The triangle beneath the consensus sequence indicates the location on the complementary strand of the *nic* site of the *oriT* of pTiR10 (38). **F.** A Neighbor-Joining tree constructed using the data from the MAFFT alignment of the *oriT*. Bootstrap values located next to the branches are from 1000 replicates. The asterisks (*) indicate plasmids that are known to be conjugative. Plasmids with Group II organization of the *rep* and Class I transfer genes are underlined.

Group I-like clade, including pTi-SAKURA, pTiS4, pTiBo542, pCB782 and pOV14c were more distantly related to their orthologs in the Group I clade in comparison with the other proteins analyzed (Figure 3.6 B). Examining the trees for the two nopaline-type Ti plasmids, the cladograms for TraH and TraB, like the trees for TraM and TraR, group pTi-SAKURA with pTiC58 (Figure 3.6 F and E, and Figure 3.3 A and B). However, the trees constructed for TraD and TraC, like the tree of TraG, indicate that these proteins from pTiC58 are most closely related to the orthologous proteins of pAtK84b, while those of pTi-SAKURA are on a separate branch (Figure 3.6 B and C, and Figure 3.5 B). The cladogram constructed for TraF has the protein from pTiC58 grouping closely with pAtS4c and more distantly grouping with that of pTi-SAKURA (Figure 3.6 D).

3.5.8 The *oriT* DNA sequences do not divide into clades based on Group I or Group II plasmid organization

TraA initiates conjugative transfer by introducing a single-strand nick (38) at the origin of transfer (*oriT*) site (Table 1.1). This *cis*-acting nucleotide sequence is

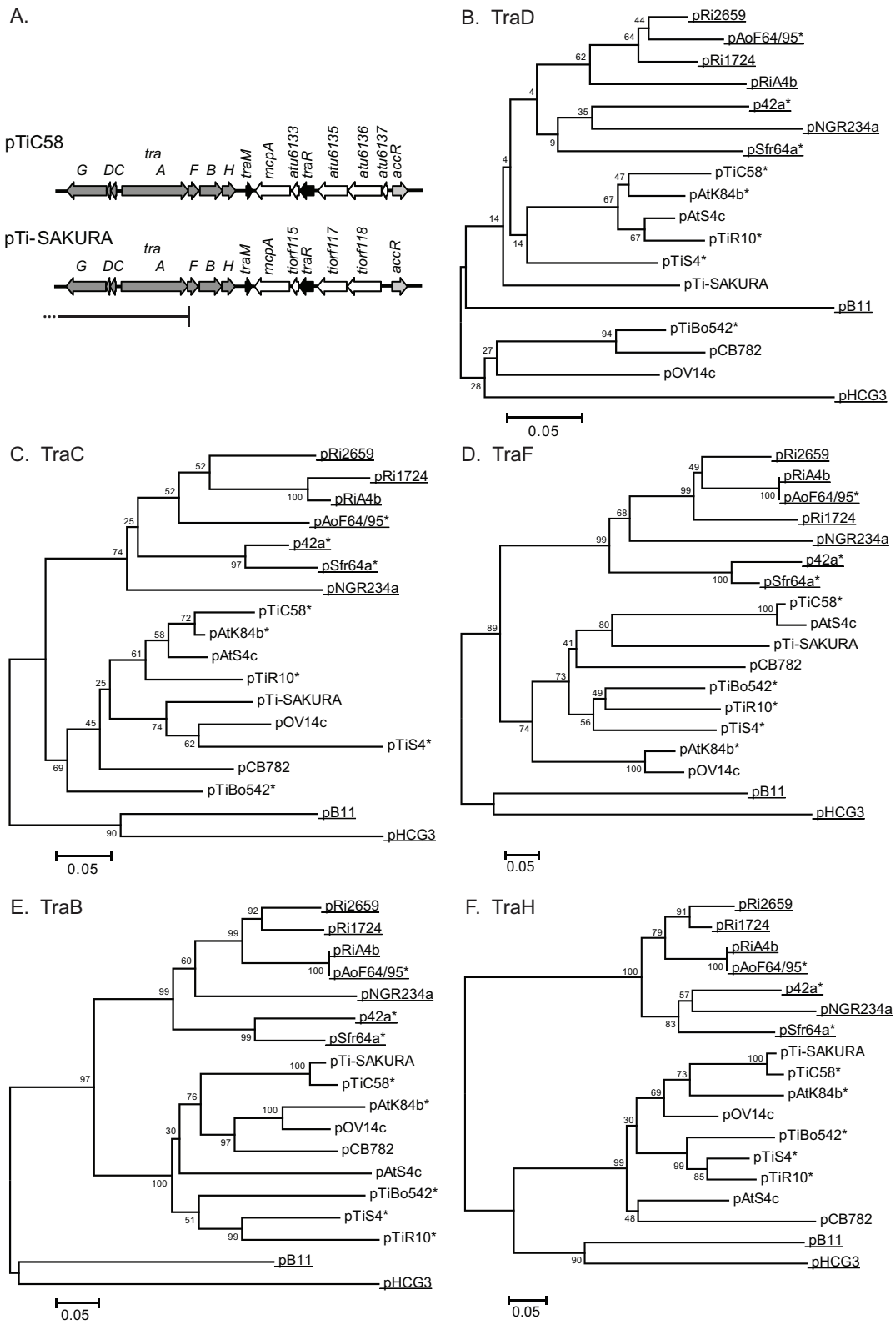

A.
pTiC58
tra
G DC A F B H traM mcpA atu6133 traR atu6135 atu6136 atu6137 accR
pTi-SAKURA
tra
G DC A F B H traM mcpA tiorf115 traR tiorf117 tiorf118 accR
B. TraD
pRi2659
pAoF64/95*
pRi1724
pRiA4b
p42a*
pNGR234a
pSfr64a*
pTiC58*
pAtK84b*
pAtS4c
pTiR10*
pTiS4*
pTi-SAKURA
pB11
pTiBo542*
pCB782
pOV14c
pHCG3
0.05
C. TraC
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pSfr64a*
pNGR234a
pTiC58*
pAtK84b*
pAtS4c
pTiR10*
pTi-SAKURA
pOV14c
pTiS4*
pCB782
pTiBo542*
pB11
pHCG3
0.05
D. TraF
pRi2659
pRiA4b
pAoF64/95*
pRi1724
pNGR234a
p42a*
pSfr64a*
pTiC58*
pAtS4c
pTi-SAKURA
pCB782
pTiBo542*
pTiR10*
pTiS4*
pAtK84b*
pOV14c
pB11
pHCG3
0.05
E. TraB
pRi2659
pRi1724
pRiA4b
pAoF64/95*
pNGR234a
p42a*
pSfr64a*
pTi-SAKURA
pTiC58*
pAtK84b*
pOV14c
pCB782
pAtS4c
pTiBo542*
pTiS4*
pTiR10*
pB11
pHCG3
0.05
F. TraH
pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pNGR234a
pSfr64a*
pTi-SAKURA
pTiC58*
pAtK84b*
pOV14c
pTiBo542*
pTiS4*
pTiR10*
pAtS4c
pCB782
pB11
pHCG3
0.05

Figure 3.6

Figure 3.6 (cont.) TraD, TraC, TraB, TraF and TraH cladograms yield similar Group II clades, but plasmids with the Group I organization show more variability between proteins. A. The organization of the *tra* region of pTiC58 and pTi-SAKURA. The *tra* genes are in medium grey and the genes for QS regulation, *traM* and *traR*, are in black. *accR*, which regulates transcription of the *arc* operon, including *traR* is light grey. The bar beneath pTi-SAKURA represents the region of a putative recombinational alteration. Neighbor-Joining trees constructed from MAFFT alignments from the 18 plasmids are shown for the following proteins: **B.** TraD, **C.** TraC, **D.** TraF, **E.** TraB, and **F.** TraH. Bootstrap values from 1000 replicates are located next to the branches. The evolutionary distances were computed with the Poisson correction. The asterisks (*) indicate plasmids known to be conjugative, while the underscores denote plasmids with Group II organization of the *rep* and Class I transfer genes.

located between the *traAFBH* and *traCDG* operons, and the core DNA sequence (Figure 3.5 E) is related to the *oriT* of the IncQ plasmid RSF1010 (41). An alignment of the nucleotide sequences of the known and putative *oriT* sites from the 18 plasmids shows conserved DNA sequences, especially at the putative *nic* site indicated by the open triangle in Figure 3.5 E. Although the cladal structure of the TraA, TraG, and TrbE proteins from the 18 plasmids correspond precisely to their organizational group, the nucleotide sequences of the putative *oriT* sites do not; the sequences of Group I and Group II plasmids intermingle within the tree (Figure 3.5 F).

3.5.9 Recombinant plasmids with cloned *oriT* regions from Group I and Group II plasmids are mobilizable by strains harboring plasmids from like and unlike groups

That the *oriT* sequences are highly conserved (Figure 3.5 E) suggests that a given *cis*-acting site may be recognized by the relaxosome components from both clades. To test this hypothesis, we determined if different *oriT* inserts could be processed by the Dtr systems from cognate and noncognate plasmids. The three pRG970b-based *tra* box I clones from pTiC58, pAoF64/95 and pRi1724 described previously also encode the respective *oriT* sites. The three *oriT* vectors were transformed into

two transfer-constitutive donors, NTL4(pTiC58Δ*accR*) a representative of the Group I clade (8), and NTL4(pAoF64/95Δ*mrtR)*, from the Group II clade (unpublished data). We then determined whether these transfer-constitutive donors could mobilize each *oriT* plasmid to the recipient strain, *A. tumefaciens* C58C1RS.

Both donors mobilized all three of the *oriT* vectors (Table 3.4). Unsurprisingly, both mobilized the vectors containing their cognate *oriT* most efficiently (Table 3.4). Moreover, the donor harboring pAoF64/95 mobilized the plasmid containing $oriT_{\text{pTiC58}}$ almost as efficiently as its cognate *oriT*. Although the Ao plasmid-containing donor mobilized the $oriT_{\text{pRi1724}}$ plasmid, the efficiency of transfer dropped by about one order of magnitude in comparison to transfer of a plasmid with its own *oriT* region (Table 3.4). Donors harboring pTiC58 mobilized both $oriT_{\text{pAoF64/95}}$ and $oriT_{\text{pRi1724}}$ at similar efficiencies, but at frequencies approximately 70-fold lower in comparison to mobilization of a vector with the cognate *oriT* (Table 3.4).

3.5.10 The RepABC plasmid replication and partitioning system, while adjacent to the *trb* operon, is more highly divergent than the QS and transfer systems

All 18 plasmids included in this study initiate replication from a *repABC* system that is conserved in the majority of large plasmids of the α-proteobacteria. In the known Class I elements, the *repABC* operon is invariably adjacent and divergently oriented to the *traI/trb* operon (Figures 3.1 and Figure 3.2). Moreover, in representatives from both organizational types transcription of the *repABC* operon, and concomitant increase in plasmid copy number, is positively controlled from the upstream *tra* box III by TraR in a quorum-dependent manner (143, 160, 181). Given the influence of QS on expression of the *rep* genes (143, 160, 181), and the location and importance of the *rep* system for stable maintenance of the plasmid, we assessed all three Rep proteins for evolutionary relatedness as we did with the proteins of the QS and conjugative transfer systems. Even though the *repABC* genes are adjacent to the

Table 3.4 *oriT* regions are recognized by cognate and noncognate *trans*-acting transfer functions

Tra^{C} plasmid[a]	*oriT*[b]	Mobilization frequency[c]
pTiC58Δ*accR*	pTiC58	2.89×10^{-3}
pTiC58Δ*accR*	pRi1724	4.25×10^{-5}
pTiC58Δ*accR*	pAoF64/95	4.55×10^{-5}
pTiC58Δ*accR*	None	6.30×10^{-6}
pAoF64/95Δ*mrtR*	pTiC58	1.03×10^{-3}
pAoF64/95Δ*mrtR*	pRi1724	3.53×10^{-4}
pAoF64/95Δ*mrtR*	pAoF64/95	2.73×10^{-3}
pAoF64/95Δ*mrtR*	None	$< 10^{-7}$

[a] All in *A. tumefaciens* strain NTL4.

[b] The *traA-C* intergenic region, including the *oriT* region of three plasmids cloned in pRG970b and placed *in trans* to the tra^{C} strain.

[c] Expressed as the number of transconjugates recovered per input donor cell. Each cross was performed in duplicate or triplicate and the values presented represent the mean of each cross.

conjugative transfer genes in these plasmids, the RepA, RepB and RepC proteins do not divide into distinct clades corresponding to organizational Groups I or II (Figure 3.7 A, B and C). Furthermore, although the cladograms of RepA and RepB are mostly congruous with each other, the tree for RepC is not (Figure 3.7). Despite their close linkage, the cladograms indicate that RepC is evolving independently from the RepAB pair.

3.5.11 The QS and transfer proteins are coevolving with each other but separately from the RepA, RepB and RepC proteins

The QS proteins and the conjugative transfer proteins, with the possible exception of TrbK, fall into two major clades that are consistent with the type of plasmid organization. If the protein sets within each plasmid are coevolving, in comparison two proteins should occupy a similar position on the phylogenetic trees in relation to the other branches. However, if the two proteins in question occupy very

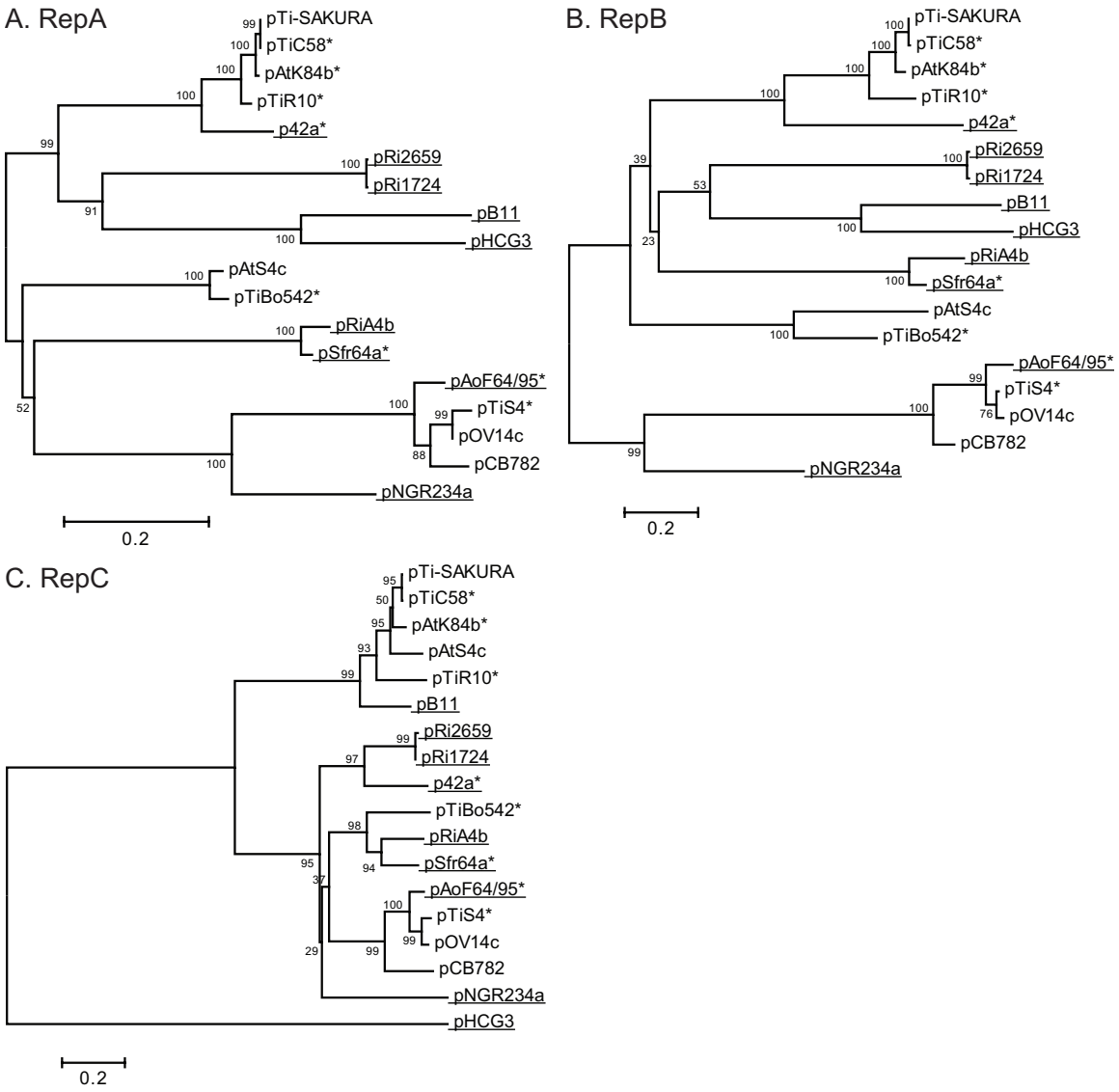


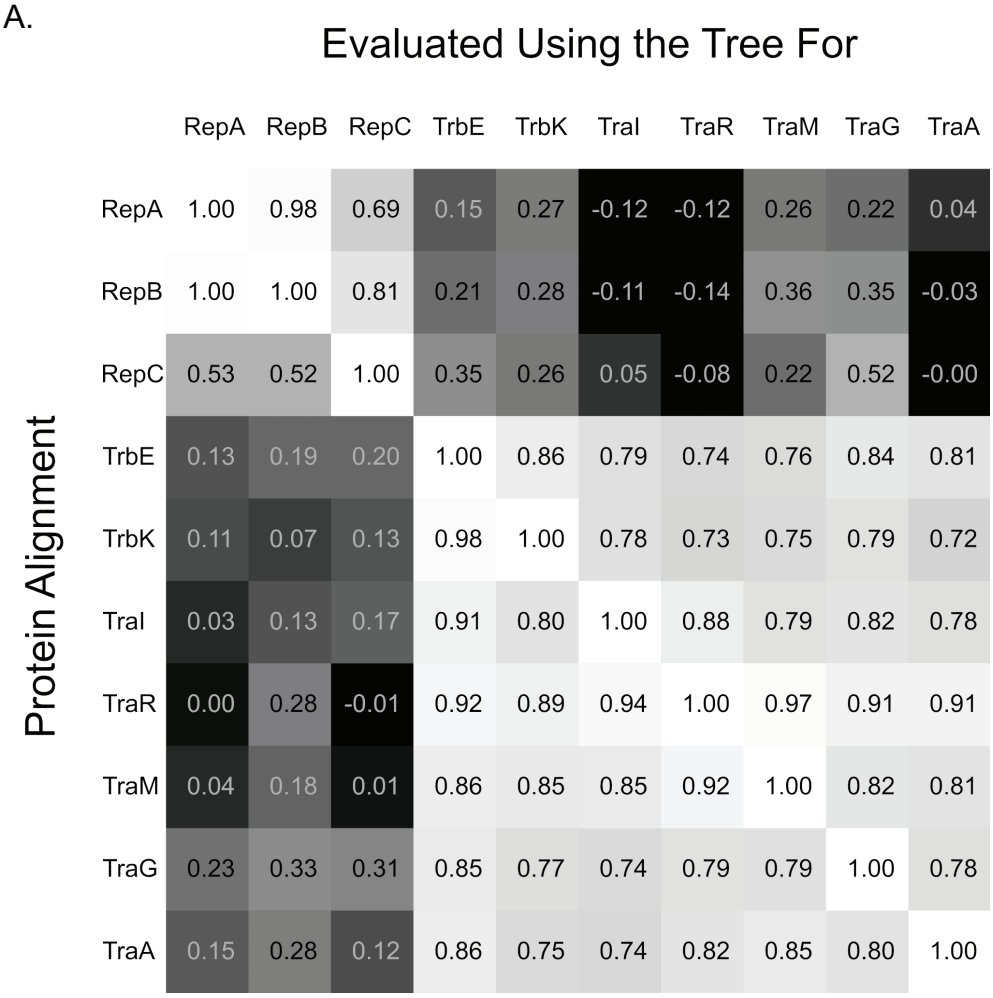
Figure 3.7 The Rep protein sequences do not divide based on plasmid organization. Trees were constructed from MAFFT alignments of **A.** RepA, **B.** RepB and **C.** RepC proteins using the Neighbor-Joining methodology. Evolutionary distances were estimated using the Poisson correction implemented in MEGA 6. Underlined plasmids have Group II organization. Asterisks (*) indicate plasmids that are known to be conjugative.

different positions on the cladograms in relation to the other branches, then the two proteins from the same plasmid have not evolved together. We assessed the

likelihood of how well the phylogenies agreed between protein sequences by comparing how well the data for one protein set would fit on a tree constructed with the data from a second protein set. Using the formula described in materials and methods, each comparison between protein data set and tree was given a score. If the trees are identical, the score is 1.00 and the heat map color is white (Figure 3.8 A). The lower the level of relatedness between a protein data set and the tree for a different protein translates to a lower number and a darker color on the heat map. The trees of the QS and transfer proteins are fairly compatible with each of the QS and transfer protein data sets (Figure 3.8 A, columns). The data for TraR indicate that the trees and protein alignments are most compatible with the trees and alignments of TraM and TraI (Figure 3.8 A, TraR rows and columns). Although the data for the TraM proteins can be fitted to the optimal trees constructed from each protein data set (Figure 3.8 A, TraM row), this tree best fits with the TraR protein alignment (Figure 3.8 A, TraM column). The TrbK protein alignment fits best with the tree constructed with TrbE (Figure 3.8 A, TrbK row). The TrbK tree, however, fits best with TraR, TrbE and TraM, and slightly less well with the remaining QS and transfer proteins alignments (Figure 3.8 A, TrbK column).

Unlike the QS and transfer proteins RepA and RepB share nearly identical optimal tree constructions but differ from any of the other trees. However, the RepC protein data set does not fit well with any of the other protein data sets, including RepA and RepB (Figure 3.8 A, RepC row). Among all of the other proteins the RepC tree fits best with the RepB protein data set followed by the RepA protein alignment (Figure 3.8 A, RepC column). Although the RepC protein alignment fits best with the RepA and RepB trees, the values are low (Figure 3.8 A, RepC row).

A.


Evaluated Using the Tree For
Protein Alignment
RepA RepB RepC TrbE TrbK TraI TraR TraM TraG TraA
RepA 1.00 0.98 0.69 0.15 0.27 -0.12 -0.12 0.26 0.22 0.04
RepB 1.00 1.00 0.81 0.21 0.28 -0.11 -0.14 0.36 0.35 -0.03
RepC 0.53 0.52 1.00 0.35 0.26 0.05 -0.08 0.22 0.52 -0.00
TrbE 0.13 0.19 0.20 1.00 0.86 0.79 0.74 0.76 0.84 0.81
TrbK 0.11 0.07 0.13 0.98 1.00 0.78 0.73 0.75 0.79 0.72
TraI 0.03 0.13 0.17 0.91 0.80 1.00 0.88 0.79 0.82 0.78
TraR 0.00 0.28 -0.01 0.92 0.89 0.94 1.00 0.97 0.91 0.91
TraM 0.04 0.18 0.01 0.86 0.85 0.85 0.92 1.00 0.82 0.81
TraG 0.23 0.33 0.31 0.85 0.77 0.74 0.79 0.79 1.00 0.78
TraA 0.15 0.28 0.12 0.86 0.75 0.74 0.82 0.85 0.80 1.00

B.

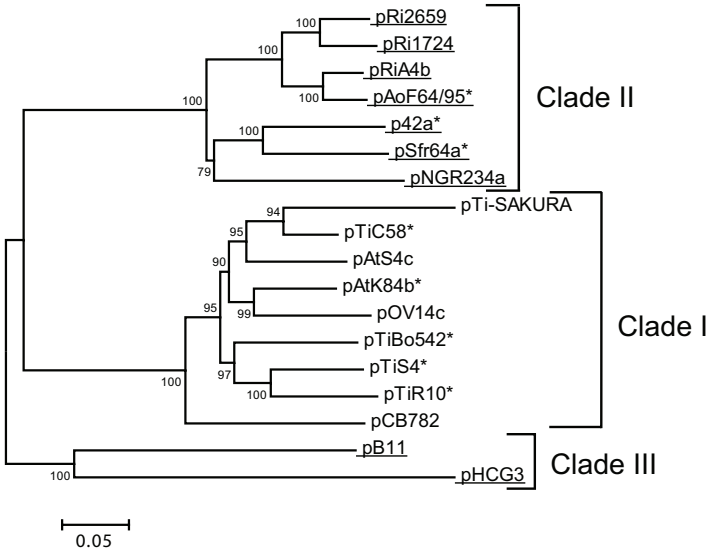

pRi2659
pRi1724
pRiA4b
pAoF64/95*
p42a*
pSfr64a*
pNGR234a
pTi-SAKURA
pTiC58*
pAtS4c
pAtK84b*
pOV14c
pTiBo542*
pTiS4*
pTiR10*
pCB782
pB11
pHCG3
Clade II
Clade I
Clade III
0.05

Figure 3.8

Figure 3.8 (cont.) Overall, the QS and transfer proteins, but not the Rep proteins, are coevolving and divide into three separate clades. A. A heat map in which alignments for ten of the core transfer, QS, and Rep proteins (vertical axis) were given a score based on how well they fit onto the trees of each protein (horizontal axis) as described in materials and methods. The shading corresponds with the values; identical tree topologies give scores of 1.00 and are white, whereas protein alignments that fit less well to a given tree, and are not coevolving have lower numbers and are darker shades of grey or are black. **B.** Neighbor-Joining tree constructed from MAFFT alignments from a concatenated sequence of the Tra, Trb, and QS proteins from the 18 plasmids. The concatenated protein contains the following proteins in order: TraR, TraM, TraI, TraA, TraG, TrbE, TrbK, TraC, TraD, TraF, TraH, and TraB. Bootstrap values from 1000 replicates are located next to the branches. The evolutionary distances were computed in Mega 6 using the Poisson correction. The asterisks (*) denote plasmids that are known to be conjugative, whereas the plasmids with a Group II plasmid organization are underlined.

3.6 Discussion

3.6.1 The quorum-sensing and transfer proteins overall form three clades associated with plasmid organization and the proteins within a particular clade are coevolving

All of the 18 *repABC* plasmids in this study that contain Class I, QS-regulated conjugative transfer systems fall into two organizational types first noted by Moriguchi *et. al*. (171). Our phylogenetic analysis indicates that evolution of the QS and transfer proteins correspond to organization; those from the Group I plasmids form one clade (Clade I), whereas the same proteins from plasmids with the Group II organization subdivide into two clades (Figures 3.1 A and 3.8 B), one from plasmids found in the family Rhizobiaceae (Clade II), the other from plasmids in the family Bradyrhizobiaceae (Clade III) (Figure 3.8 B).

Remarkably, with respect to the virulence elements of *Agrobacterium*, the organizational split correlates with function. We know of no Ri plasmid with Group I organization. Nor do we know of any Ti plasmid with Group II organization. This observation implies that all of the Ti plasmids and all of the Ri plasmids, while

sharing a common ancestor, have diverged and are evolving independently. However, among opine-catabolic plasmids, both organizational types are represented (see Table 3.2). We conclude that this "cheater" class of opine-catabolic plasmids can evolve from either Group I or Group II elements. The two Sym plasmids examined, pNGR234a and pCB782, divide between Groups I and II (Table 3.2 and Figure 3.8 B). This observation suggests that this functional group of plasmids is less constrained in its evolution as compared to the virulence elements of the agrobacteria.

The trees constructed from the proteins of pHCG3 and pB11 suggest that these two Group II bradyrhizobial plasmids have diverged from the other Group II elements and now constitute an independently evolving clade. Although only sparsely described, the existence of Class I *repABC* plasmids in these diverse and ecologically distinct bacteria indicate that members of this family of episomal elements have extended their genetic range beyond bacteria that interact with plants.

Although there is a clear phylogenic divergence of the QS and transfer proteins into three clades, consistency in the branch structures of the 12 QS and transfer proteins examined indicates that these proteins have not only evolved into separate clades, but are also coevolving in their particular plasmids within a clade (Figures 3.3, 3.5, and 3.6). The scores in the heat map analysis support this conclusion (Figure 3.8 A). The robustness of these evolutionary patterns is further illustrated by the structure of a cladogram constructed using a concatenated protein alignment of all 12 protein sequences from the 18 plasmids examined (Figure 3.8 B).

Overall, components of the QS and transfer systems that interact with each other or lie in the same operon show the highest degree of coevolution. For example, TraM and TraR interact with each other, and for both Group I and Group II plasmids, the *traR* and *traM* genes are always located near one another (Figures 3.1 and 3.2).

Concomitantly, these two proteins exhibit largely similar trees and show a high degree of coevolution in the heat map (Figures 3.3 and 3.8 A).

3.6.2 The highly conserved *oriT* regions can be recognized and nicked by components of the relaxosome from both clades

That there is cross-cladal functioning between the *trans*-acting *tra* components and *cis*-acting *oriT* sites (Table 3.4) raises the possibility that induction of transfer by a specific signal of one plasmid may stimulate transfer from that donor of a coresident, noninduced plasmid with an appropriate *oriT* region. Although cotransfer of plasmids has been described in both *Rhizobium* and *Agrobacterium* species, most studies have concluded that this phenomenon is likely the result of plasmid cointegration (18, 185). However, cotransfer of a noninduced megaplasmid by *trans*-mobilization has been reported (240). The range of mechanisms involved in cotransfer of these large plasmids merits further study.

3.6.3 TraR proteins have DNA binding and dimerization functions that are conserved within, but not between clades

Our *in vivo* analysis showing that TraR-dependent promoters are activated by only cognate and closely related TraR orthologs is consistent with the *in silico* results that the QS and transfer proteins are coevolving with each other within, but not between clades (Figures 3.4 and 3.8 A). Our results extend those of He, *et. al*. (104) showing that TraR of pNGR234a, with a Group II organization, can activate transcription of at least two of its cognate promoters, but does not activate the same promoters from pTiR10, a Group I-type plasmid (104). Moreover, $TraR_{pTiR10}$ activates a cognate TraR-dependent promoter but not the orthologous promoter from pNGR234a (104). Coupled with our observation that TraR proteins from the two major clades cannot efficiently cross-dimerize, our results suggest that both DNA binding and dimerization domains of TraR proteins are structurally and functionally conserved within, but not between the clades.

3.6.4 $TraR_{pRi1724}$ functionally differs from other TraR proteins in the *repABC* family of plasmids with Class I transfer systems

Gene activation by TraR of pRi1724 differs remarkably from that of the archetypical system. The TraR proteins from pTiC58 and pAoF64/95 strongly activate their cognate *traA* promoters, but only in cells grown with the appropriate quormone (Figure 3.4 B). TraR from pRi1724, on the other hand, activates its cognate *traA* promoter, and also that from pAoF64/95, but only in cells grown in the absence of the QS signal (Figure 3.4 A). Three lines of evidence indicate that the anomalous behavior of $TraR_{pRi1724}$ is a function of the activator, and not of its *tra* box binding site. First, the *tra* box I sequences of pRi1724 and pAoF64/95 are identical (Figure 3.3 E). Second, $TraR_{pAoF64/95}$ activates expression from the *traAFBH* promoter from both pAoF64/95 and pRi1724 in a quormone-dependent manner (Figure 3.4 B). Third, $TraR_{pRi1724}$ activates transcription of these two promoters only in the absence of the autoinducer (Figure 3.4 A). That the amino acid sequences of the recognition helices of $TraR_{pRi1724}$ and $TraR_{pAoF64/95}$ are strongly conserved suggests that the anomalous properties of $TraR_{pRi1724}$ are due to alterations in regions of the protein other than those involved in quormone binding or DNA site recognition.

The anomalous activity of $TraR_{pRi1724}$ could be a result of random mutational events or could represent a regulatory divergence where transfer is induced only when the acyl-HSL-producing population is low. Other LuxR homologs such as EsaR of *Pantoea* spp. and YenR of *Yersinia enterocolitia* bind to their target DNA sequence in the absence, and not the presence, of the acyl-HSL cognate to the system. Although apo-EsaR represses transcription of the target genes when the population density is low (9, 165), apo-YenR activates transcription of a target gene, *yenS*, at low population densities, a regulatory strategy called quorum-hindering (242).

3.6.5 The RepA, RepB, and RepC proteins do not coevolve with the transfer and quorum-sensing proteins

RepA, RepB, and RepC comprise a well-characterized family of replication proteins that, in the 18 Class I plasmids included in this study, are located adjacent and divergently oriented to the *traI*/*trb* operon. The fact that the arrangement of the *rep* genes and the *traI*/*trb* operon is obligatory in plasmids with the Class I transfer system raises the possibility that the *rep* system is coevolving with the genes of the adjacent transfer systems and perhaps even the QS regulatory system. This proved not to be the case; the trees constructed for the RepA, RepB, and RepC proteins do not divide based on plasmid organization (Figure 3.7). Moreover, none of the Rep proteins are coevolving with the *traI*/*trb* operon, despite their close physical linkage as divergently oriented operons (Figures 3.3, 3.5 and 3.7). This divergent evolution of the *rep* and transfer genes is especially striking considering that the *repABC* and *traI*/*trb* operons share a region containing promoters and *tra* boxes, allowing TraR-mediated regulation of transcription of not only the genes for transfer, but also the gene system for plasmid replication and partitioning (143, 160, 181).

Our analyses showing that RepA and RepB proteins coevolve with each other but do not strongly evolve with RepC confirm previous studies of the coevolutionary relationships of these three proteins [reviewed in (188)]. Although the RepABC proteins have been extensively studied, to our knowledge, ours is the first report comparing the phylogenies of the RepA, RepB, and RepC proteins with the proteins of the conjugative transfer and QS systems encoded by these plasmids. This independent evolution makes sense considering that genetically active components within the *repABC* operon determine plasmid incompatibility (7, 23, 26, 27, 156, 157, 195, 250). This property of plasmids is often determined by the degree of relatedness of the replication and partitioning functions of the elements (176). The problem of incompatibility is of considerable interest; some isolates, such as *Rhizobium etli* strain CFN 42, stably maintain as many as six different *repABC*

replicons (93). Clearly the components of the replication system are actively evolving to generate different incompatibility properties. Uncoupling the evolution of the replication system from the transfer system provides these plasmids with the ability to transfer to and be stably maintained in a variety of strains, even those harboring other *repABC* replicons.

The three Rep proteins, as well as a small RNA and *cis*-acting sites within the operon are involved in plasmid incompatibility (23, 184, 188, 195). pTiC58, pAtK84b, and pTiR10 all belong to the IncRh-1 group (40, 111, 166). Strikingly, for the trees constructed with the RepA, RepB, and RepC protein sequences, these plasmids group within the same subclade (Figure 3.7). The tartrate-catabolism plasmid of *A. vitis* strain S4, pAtS4c, belongs to the IncRh-2 class (224), while the coresident Ti plasmid, pTiS4, belongs to the IncRh-4 class (223). Consistent with this compatibility, the RepA, RepB, and RepC proteins of pTiS4 and pAtS4c are members of separate subclades. These two examples provide phylogenetic support for the role of divergent evolution of these proteins in determining the ability of the *repABC* plasmids to coexist in the same host.

3.6.6 Some of these plasmids are highly homologous and have shared evolutionary histories

Several of the plasmids examined in this analysis share significant regions of synteny. For example, pRi1724 and pRi2659 are highly homologous over their entire lengths (171). Both of these Ri plasmids were isolated from biovar 1 strains of *A. rhizogenes*, one from a diseased melon plant in Japan and the other from a diseased cucumber plant in the United Kingdom. For nearly all of the 15 protein sequences analyzed, including RepA, RepB, and RepC, the proteins of pRi1724 and pRi2659 are related most closely to each other. Considering that overall the Rep proteins evolve separately from the transfer and QS proteins, the fact that these two

sets of proteins of pRi1724 and pRi2659 are evolving together indicates that the two plasmids share a very recent common ancestor.

Similarly, pTiC58 and pTi-SAKURA, which are both nopaline-agrocinopine-type Ti plasmids share syntenic regions that are highly homologous (94, 222). Despite the apparent recombination-generated divergence in the region around *traF*, the QS, conjugative transfer, and Rep proteins of pTiC58 most closely resemble those of pTi-SAKURA, indicating that these two plasmids share a recent common ancestor. That such a recombination even has occurred is not surprising, plasmids in the *repABC* family are known to recombine, often by forming and resolving cointegrates (18, 122, 159, 255).

3.6.7 Evolution of the Class I-*repABC* family of plasmids is complex

The differences in organization of the conjugative transfer genes of the Group I and Group II plasmids and the chimeric nature of these large plasmids make the evolutionary study of these extrachromosomal elements intriguing and complex. The two types of plasmid organization suggest that expansion of a conceptual core plasmid containing the QS, *rep*, and transfer genes (pCORE, Figure 3.9) occurs by inserting new DNA primarily in two locations. The first and perhaps preferred location occurs between the *repABC* and *traCDG* operons (Figure 3.9, Site A). The second region (Figure 3.9, Site B) is located downstream of the *trb* genes and separates this operon from the *traR-traM-tra* cluster. Both Group I and II plasmids exhibit insertions in variable region A, while Group I plasmids have a second variable region inserted into Site B (Figure 3.9). Intriguingly, Ti plasmids, which all show Group I organization, differ with respect to which of the two variable regions encoding loci important for tumor induction are located. For example, the segment of DNA encoding the T-region and the *vir* regulon of the Octopine-type Ti plasmids is inserted into Site A, whereas the analogous segment of pTiC58 is inserted in Site B. Of significance to the evolution of regulation in the Group I Ti plasmids, insertions

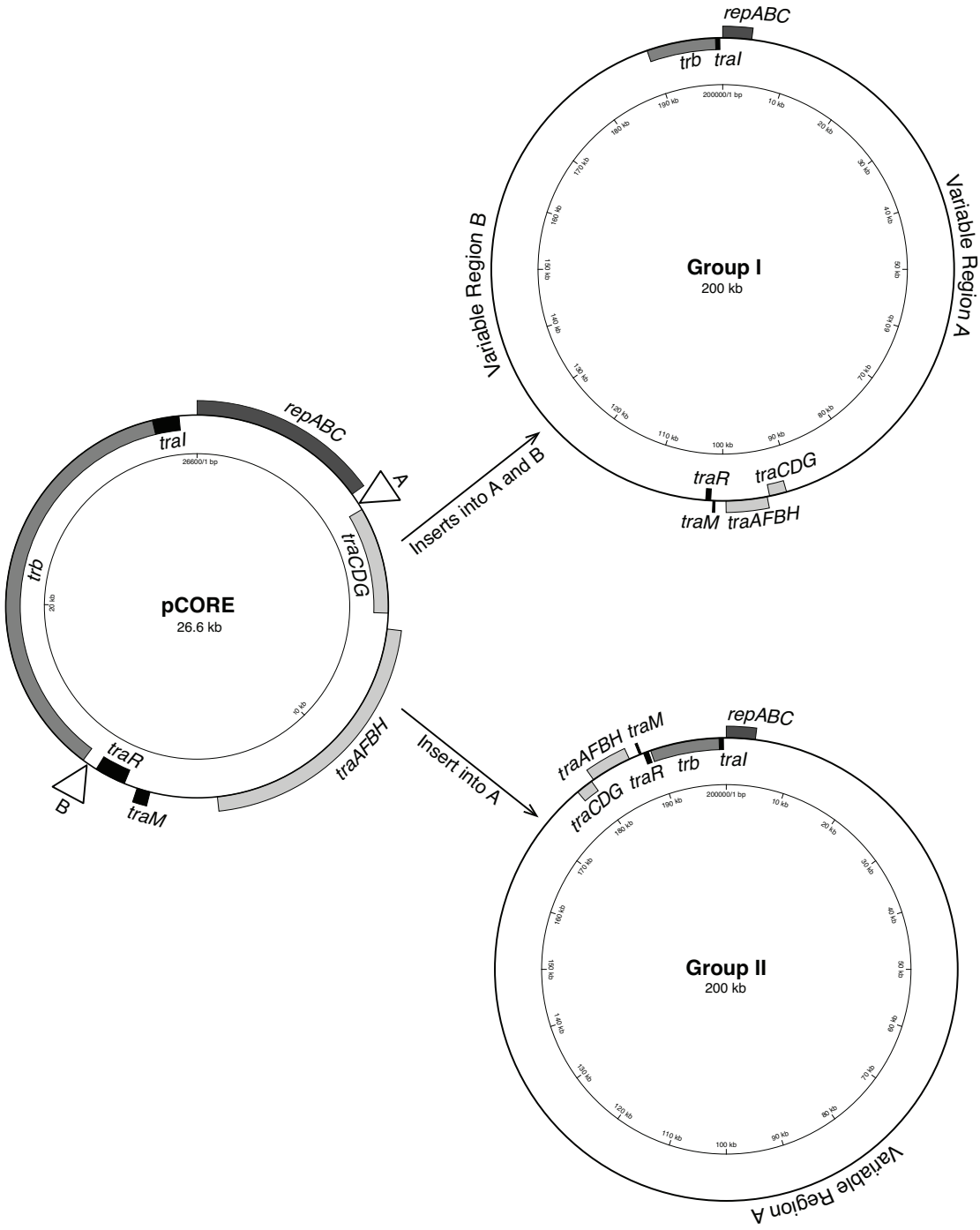


Figure 3.9 Acquisition of additional DNA by Class I-*repABC* plasmids occurs at two favored locations. A conceptual *repABC* plasmid, pCORE, containing the core Class I transfer genes (medium grey for Mpf genes and light grey for Dtr genes), the QS genes *traR*, *traM* and *traI* (all in black) and the *rep* operon (dark grey). The triangles labeled A and B mark the sites at which additional sequence is found in the two organizational types, Group I and Group II, of plasmids.

into the B site provide a mechanism by which *traR* can be fused to a gene system regulated by the conjugative signal. Insertions into the other two regions, between the two *tra* operons and between *traI*/*trb* and *repABC*, most likely is restricted by their richness in *cis*-acting promoter and regulatory sequences.

Despite the organizational differences and the role of recombination and horizontal gene transfer in increasing genetic diversity, overall our analysis indicates that the genes of the *tra* and *trb* regions of a particular plasmid are coevolving. Additionally, the orthologous genes shared by these plasmids within a clade coevolve and retain a level of functionality within, but not between clades. Furthermore, we conclude that the QS proteins overall are coevolving with the transfer system.

Although the evolution of these plasmids may be restricted to the two organizational groups, it is most probable that additional clades will emerge as more sequence becomes available. Even now, the tree structures of pHCG3 and pB11 suggest that these two plasmids have evolved into a new Group II clade (Figure 3.8 B, Clade III). Evolution of these plasmids is of particular importance to the order Rhizobiales for several reasons. First, the *repABC* family of plasmids exhibit an extended conjugative and replicative host-range among the α-proteobacteria. Second, the *repABC* elements have a large genetic carrying capacity and the genes encoded on these replicons often confer defining characteristics to the bacteria that harbor them. Third, quite clearly some of the *repABC* plasmids are evolving into chromids in a range of genomes within the order Rhizobiales (4, 101, 139). Considering the potential for conjugative transfer and dissemination of these Class I plasmids, the large genetic carrying capacity, their propensity to recombine, and the extended host range encoded by *repABC* lend importance to the study of the evolution and interactions of this group of plasmids, and the bacteria that harbor them.

Chapter 4: Conclusions

4.1 The *repABC* family of plasmids with Class I transfer systems is important to the biology of the bacteria that harbor them

The *repABC* plasmids found in the Rhizobiales are important to the biology of the bacteria that harbor them for several reasons. First, replicons that replicate and segregate using RepA, RepB, and RepC can stably carry large quantities of genetic information, and some of these elements are, or are evolving into, secondary chromosomes, now called chromids (101). Moreover many of these plasmids encode functions, such as virulence, that define the bacteria that harbor them. A subset of *repABC* plasmids encode genes for horizontal transfer of the plasmid from one bacterium to another [(89) and reviewed in (59)]. The most well-studied of these is the Class I transfer system that is encoded by the *tra* regulon. In all cases known to date, the *tra* regulon, composed of the two *tra* operons and the *traI*/*trb* operon, is regulated by a quorum-sensing relay involving an activator, TraR, an antiactivator, TraM, and a ligand synthase, TraI.

4.2 There are two distinct genetic arrangements of plasmids with Class I transfer systems

Among the *repABC* family plasmids with Class I conjugative transfer systems all genes involved in transfer and regulation of transfer are conserved. However, Moriguchi *et. al*. first noted that there are two distinct genetic arrangements of the genes involved in transfer and replication (171). Both groups invariable have the *traI*/*trb* operon adjacent and divergently oriented to the *repABC* operon. In Group I plasmids, the two *tra* operons, along with *traR* and *traM*, are separated from the *trb* operon, often by more than 60 kb. Unlike those of the Group I, in plasmids with Group II organization the *repABC*-*trb* region is contiguous with the two *tra* operons, and *traM* and *traR* are located between the last gene of the *traAFBH* operon and the end of the *traI*/*trb* operon. In cases where transfer of Group I and Group II plasmids

has been demonstrated, TraR directly activates the two *tra* operons along with the *traI*/*trb* operon [reviewed in (59)] . In Group I plasmids found in the agrobacteria, transfer is inducible by a specific opine because *traR* is a member of an operon that is inducible by the conjugative opine (82, 177, 178, 190). Unlike the Group I plasmids, *traR* of Group II plasmids is monocistronic (44, 171, 257). The two different modes of organization of the QS and transfer genes suggested to us that relevant core genes in plasmids with Group I and Group II organization are evolving independently.

4.3 The transfer and quorum-sensing proteins along with the *tra*-box sequences from plasmids in the Rhizobiales form three major clades

In our analysis of the protein sequences of the *tra*, *trb*, and adjacent *rep* genes from 18 different plasmids, nine with the Group I organization and nine with Group II organization, the gene sets all form three major clades that are consistent with gene organization. The proteins from Group I plasmids form a separate tree that we call Clade I. Proteins from plasmids with Group II organization divide into two additional clades, Clades II and III. Clade II contains sequences from plasmids found in the Rhizobiaceae, while Clade III contains sequences from plasmids isolated from members of Bradyrhizobiaceae. Based on phylogenetic and heat map analyses, it is evident that the QS and transfer proteins within a particular plasmid are coevolving. Moreover the core genes are coevolving within each group but are divergent between the two groups. Functional analyses are consistent with the organization-associated cladograms. TraR proteins can activate transcription from *tra* box-containing promoters within, but not between, clades. Moreover TraR proteins can dimerize within, but not between, clades. The analysis of promoter activation and TraR-dimerization are consistent with phylogenetic analysis of TraR and the *tra* box DNA sequences. This clearly indicates that at least the QS and transfer genes have diverged and that this divergence coincides with the two different organizations of these genes on *repABC* plasmids.

4.4 *oriT* sequences are highly conserved and can be mobilized utilizing the transfer functions of plasmids from either clade

While the protein sequences of the transfer and QS proteins and the *tra* box sequences indicate that there is a phylogenetic division between plasmids with Group I and Group II organization, the *oriT* sequences are highly conserved and there is no clear cladal division of these DNA sequences based upon plasmid organization. Functional analysis is consistent with the phylogeny; plasmids with cloned *oriT* regions can be mobilized using the transfer systems of plasmids with either Group I or Group II organization. This close relatedness suggests that the *oriT* sequences of both Group I and Group II plasmids are sufficiently conserved to be recognized by the *oriT* recognition and processing relaxosome proteins of both groups of plasmids. Such sequence conservation raises the interesting possibility that transfer of a Class I replicon could be mediated by the *trans*-acting *tra* system of a coresident Class I plasmid. In this manner *trans*-mobilization of plasmids could increase the amount of horizontal gene transfer of these elements between bacteria. Cotransfer of plasmids has been documented, but most studies conclude that this results from cointegration of two plasmids followed by transfer of the cointegrate, rather than true *trans*-acting mobilization (18, 185). However, *trans*-mobilization of at least one plasmid, pLPU83a, has been reported (240).

The *oriT* sequences of the Class I transfer systems are related to those of IncQ plasmid such as RSF1010 and to IncP-1α plasmids, such as RP4 (41). While an RSF1010 derivative can be mobilized by the transfer system of RP4, a vector containing the *oriT* region from RP4 or the *oriT* region from RSF1010 cannot be mobilized by a Ti plasmid transfer system (41). However a clone containing the *oriT* of RSF1010 can be mobilized by the Ti plasmid MPF system if the coupling factor, $TraG_{RP4}$, is expressed (98). $TraG_{RP4}$ cannot substitute for $TraG_{Ti}$ to mobilize the Ti plasmid (98). These patterns of cross functionality indicate that the coupling factor is specific for the relaxosome and that the interaction of TraG with the MPF system

is less specific. Taken together with the results reported by us, these observations open the possibility that relaxosomes of both Group I and Group II plasmids in the Rhizobiales can be recognized by TraG from either group of plasmids, a hypothesis yet to be tested.

4.5 The replication and partitioning system do not evolve with the quorum-sensing and transfer systems

Although the transfer and QS systems have diverged into three major clades closely associated with plasmid organization, the genes of the replication and partitioning system evolve independently from those involved in transfer and its regulation. The RepA and RepB proteins coevolve with each other, but RepC evolves independently from both RepA and RepB. That the evolution of the *rep* system is unconnected to the adjacent *tra* system is interesting given that the *repABC* system confers plasmid compatibility. In theory, this allows different *repABC* plasmids encoding related Class I transfer systems to be harbored by the same bacterium. In practice, there are several examples of such plasmids existing within the same bacterium. Moreover, in at least one case a conjugative *repABC* plasmid mobilizes transfer *in trans* of a second *repABC* plasmid (240). Furthermore, in those plasmids tested TraR upregulates expression of the *repABC* operon, thereby increasing plasmid copy number (143, 160, 181). It is intriguing that TraR and the *tra* regulon are coevolving, but that TraR and the *rep* operon invariably adjacent to the *tra* regulon are not coevolving, despite the fact that TraR regulates this system. It is also remarkable that the *traI*/*trb* operon of these plasmids does not coevolve with the *repABC* operon, despite the fact that they are invariably tightly linked to each other. While the evolution of the Rep proteins has been examined in some detail [reviewed in (188)], this is the first study that has assessed the evolution of RepA, RepB, and RepC in the context of coevolution with the proteins involved in the Class I conjugative transfer and QS systems.

4.6 Evolution of the *repABC* plasmids with Class I transfer systems is complex

The analysis of the genes for transfer, QS and replication presented in this dissertation explore several other themes. Some plasmid pairs, such as pTiC58 and pTi-SAKURA, and pRi1724 and pRi2659, share large regions of homology and synteny. Clearly each set of plasmids has a common recent evolutionary ancestor. However, it is also clear that evolution of these plasmids is complex. For example, while pTi-SAKURA shares a recent common ancestor with pTiC58, it has a chimeric *tra* region. This suggests that a recombinational event has occurred that has led to a structural divergence of pTiC58 and pTi-SAKURA. Many plasmids in the Rhizobiales are chimeric and are known to recombine by forming and resolving cointegrate structures (22, 79, 92, 171, 219, 255). Conjugation imports new genetic material into the cell, and these plasmids have the potential to recombine with the plasmids already present in any particular host. This not only makes the evolution of these plasmids more complex, it also increases the genetic diversity of plasmids and the functional diversity of their host bacteria.

In all of the plasmids examined, the Class I transfer and QS genes are associated with a *repABC* replication and partitioning system that is invariably located adjacent and divergently oriented to the *traI*/*trb* operon. Based on these similarities, we can imagine a hypothetical plasmid that contains only the core transfer, QS and replication systems (Figure 3.9). While many of the *repABC* plasmids with Class I transfer systems range between 170-250 kb, the core functions are encoded by only 27 kb. Considering that there is so much excess variable DNA in the sequenced plasmids in comparison to this core, there seem to be two regions where insertions typically occur. The first of these regions, variable region A, is located between *repC* and *traH*, the last gene of the *traAFBH* operon (Figure 3.9). All Class I plasmids known to date harbor insertions in this site. Considering this site is occupied in both Group I and II elements, it seems this is the preferred location for genetic cargo for the plasmids in the Rhizobiales. Insertions in the second site, variable region B,

are located between the last gene of the *traI*/*trb* operon and *traR* and are found only in the Group I plasmids (Figure 3.9). Insertions in site B can also account for the apparently serendipitous fusion of *traR* to a genetically active gene system. Given that site B is located just upstream of *traR*, insertions into the B site provide a mechanism to fuse *traR* to an operon inducible by a particular signal, such as an opine. For example, $traR_{pTiC58}$ is the distal gene of the *arc* operon, an operon inducible by the agrocinopines (190), while $traR_{pTi15955}$ is the distal gene in an operon inducible by octopine (82) (Figure 4.1). At least two Group I plasmids harbored by species of *Rhizobium* and *Ensifer*, pCB782 and pOV14c, show a similar fusion of *traR* to gene systems potentially involved in catabolic functions. *traR* of pCB782 is located at the end of an operon that could encode catabolism of an unknown organic compound, while *traR* of pOV14c lies downstream of an operon encoding a putative ABC-type transporter (Figure 4.1). These gene organizations are of interest from two perspectives. First, linkage of *traR* to genes involved in catabolism of exogenous carbon sources opens the possibility that expression of *traR* and the induction of conjugation by exogenous environmental signals extends beyond the well-studied role of tumor-produced opines common to plasmids of the agrobacteria. Second, if such is the case, then this suggests that such plasmids have evolved a specific mechanism to couple conjugation to environmental conditions.

While the regulation of transfer has been well-documented in the Group I Ti plasmids (82, 177, 178, 190), the conjugative properties of only two Class I plasmids with Group II organization have been examined in detail (18, 44, 149, 243, 257). These two Group II plasmids, pRL1JI and p42a, exhibit two different regulatory mechanisms. p42a is constitutive for transfer because expression of *traR* is constitutive and *traM* , the TraR antiactivator, is not transcribed (18, 243). For pRL1JI, expression of *traR* is induced by a second, orphaned LuxR-type activator in response to a second acyl-HSL signal that is produced by the recipient (44, 149, 257). Considering that the research concerning induction and regulation of transfer of Group II plasmids is scant and that the two described systems differ, the

mechanisms by which bacteria harboring Group II plasmids respond to signals in the environment to move these genetic element between themselves merit further study.

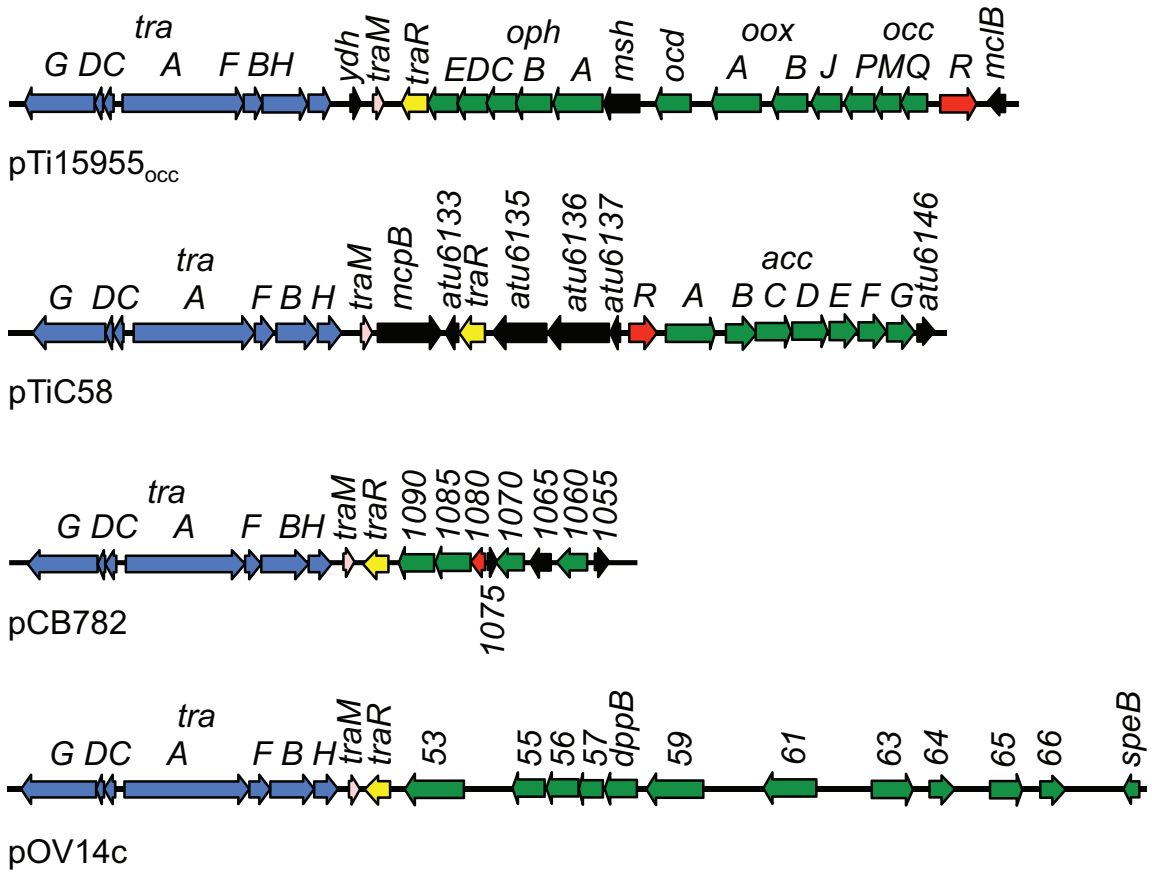


Figure 4.1 In Group I plasmids *traR* can be fused to an operon involved in catabolism or transport of an organic compound. The genes for DNA metabolism and mating pair formation are colored in blue, while *traM* and *traR* are pink and yellow respectively. Known or putative genes involved in transport and/or catabolism of organic compounds are pictured in green. Known and potential regulatory genes are colored in red. All other genes are pictured in black.

All Ti plasmids show Group I organization, whereas all Ri plasmids examined show Group II organization. This remarkable correspondence of structure and function implies that, while the Ti and Ri plasmids share core replication and transfer genes,

they arose separately and the two plasmid types are evolving independently. If such is the case, cargo DNA common to the organizational class, the genes of the *vir* regulon, for example, should show a similar evolutionary divergence. We are not aware of any comprehensive study concerning this issue. The opine catabolic plasmids can either have Group I or Group II organization indicating that these elements can evolve from either group of plasmids. One can imagine that this "cheater" class of elements evolves from the Ti or Ri virulence elements by a loss of the T-region, and the *vir* operons. Such a path could account for retention of the genes for opine catabolism. While most plasmids in species of *Rhizobium* and *Ensifer* fall into Group II, at least two Sym plasmids, pCB782 and pOV14c, exhibit Group I organization. This observation implies that the evolutionary histories of the Sym elements are less constrained than that of the Ti and Ri plasmids of *Agrobacterium*. Finally, the two representative Class I plasmids that were isolated out of bradyrhizobial strains, pHCG3 and pB11, demonstrate a Group II organization but have evolved away from the Group II elements found in the Rhizobiaceae and form a separate independently evolving linage, Clade III. Clearly, the *repABC* elements with Class I transfer systems have expanded their host range to include bacteria that do not interact with plants. Remarkably, pHCG3 of *O. carboxidovorans* carries the genes that allow this bacterium to grow autotrophically on CO and CO_2. This plasmid represents another example of the genetic plasticity of the *repABC* elements and how they evolve to confer novel traits to their bacterial host. That plasmids with Group II organization are found in both the Rhizobiaceae and the Bradyrhizobiaceae could indicate that this simpler organization is ancestral or that the Group II elements are more efficient at transferring and replicating in a variety of host backgrounds in comparison to plasmids with Group I organization.

4.7 pAoF64/95 is a Group II plasmid inducible for transfer by the opine mannopine

Our contribution to the question of how transfer responds to environmental cues in Group II plasmids with monocistronic *traR* genes took a serendipitous start. The octopine-type Ti plasmids pTi15955 and pTiR10 are virtually identical elements that encode utilization of octopine and all four of the mannityl opines. Transfer of these plasmids is inducible by octopine because *traR* is in an operon inducible by the opine (82). Later, two groups identified a dominant-negative frame shifted allele of *traR* on pTi15955 and pTiR10 called *trlR* that is located in an operon inducible by mannopine (MOP) (179, 267). Although TrlR was expressed when the cells were grown with MOP, transfer was not induced (179, 267). We reasoned that there must be a field isolate that harbors a plasmid in which MOP induces expression of *traR* and therefore transfer, and that *traR*, like *trlR*, is linked to the mannopine transport (*mot*) operon. In an assessment of a number of MOP-utilizing wild-type isolates we found five strains in which the opine induced transfer of the plasmid. These five plasmids all were highly similar so we focused on only one, pAoF64/95. This plasmid conferred on the bacterium the ability to transfer the MOP-utilization trait only when cells were grown with MOP. Sequence analysis revealed that this plasmid, while not a virulence element, shows a Group II organization of the genes involved in QS and transfer. Of considerable interest, *traR*, unlike that of the Group I plasmids, is monocistronic. pAoF64/95 is not only the first plasmid demonstrated to be inducible by MOP, but it is also the first Group II plasmid for which transfer is found to be inducible by an opine.

4.8 pAoF64/95 is an opine-catabolic plasmid

In addition to encoding all of the genes involved in QS and Class I conjugative transfer, pAoF64/95 encodes all the genes required for uptake and catabolism of three of the four mannityl opines- mannopine, mannopinic acid, and agropinic acid-

but is lacking the genes for uptake and utilization of agropine. pAoF64/95 also encodes the *acc* operon responsible for uptake and utilization of the agrocinopine opines and sensitivity to agrocin 84. Consistent with sequence analysis, strains harboring pAoF64/95, in addition to utilizing MOP, can also utilize MOA and AGA and are susceptible to agrocin 84. Considering that pAoF64/95 codes for utilization of opines but does not encode the virulence and opine synthesis functions of the Ti and Ri plasmids, this element must be primarily an opine catabolic plasmid. Remarkably, based on restriction endonuclease fragment patterns, pAoF64/95 is related to several other plasmids in the Corvallis collection and also to another opine catabolic plasmid, pArA4 of *A. rhizogenes* A4. Like pAoF64/95, pArA4 confers utilization of MOP, MOA, AGA, and agrocinopines to its bacterial host. Clearly this conserved opine-catabolic plasmid is widely disseminated among agrobacterial populations. The fact the opine catabolic plasmids are widely distributed and confer utilization of the opines produced by plants induced by virulent agrobacterial strains indicates that these plasmids likely evolved as "cheaters" by uncoupling a need to encode functions for virulence and opine synthesis in the tumor from the functions that allow their bacterial hosts to utilize opines in the rhizosphere. Therefore these plasmids can confer the selective advantage of opine utilization to their hosts without carrying the genetic real estate that encodes the *vir* regulon or the T-regions with their opine synthesis genes. This allows the bacteria harboring the "cheater" plasmids to reap the benefits of carbon sources from the tumor environment that was induced by another strain of *Agrobacterium*.

4.9 The quorum-sensing system of pAoF64/95 is functional

Much of what is known about regulation of transfer of Class I plasmids has come from studies of the Group I Ti plasmids. In this system, opines induce transfer by regulating expression of *traR*. Considering that the inducing signal and complete sequence of pAoF64/95 is known and that regulation of Group II plasmids requires further research, pAoF64/95 is an excellent choice to investigate how Group II

plasmids regulate transfer. Not only is the transfer system of pAoF64/95 conserved, but the genes for QS regulation- *traR*, *traI*, and *traM*- also are conserved. Analysis of strains harboring pAoF64/95 and cosmid clones encoding $traI_{pAoF64/95}$ reveal that $TraI_{pAoF64/95}$ produces 3-oxo-C8-HSL, the known quormone for QS regulation of Class I plasmid transfer. We also constructed and analyzed mutations in both *traM* and *traR* of pAoF64/95. *traM* mutants are constitutive for conjugative transfer, indicating that the role of TraM as an antiactivator is conserved. *traR* mutants of pAoF64/95 fail to transfer, even when grown with MOP, indicating that $TraR_{pAoF64/95}$ is an activator of conjugative transfer. However, there is a significant difference between the regulatory system of pAoF64/95 in comparison to that of the Ti plasmids. In all Ti plasmids studied to date, a mutation in *traR* is complementable by a recombinant clone expressing the wild-type allele. Moreover, expression of the cloned *traR* genes obviates the need for the conjugative opine (82, 177, 189). However, although a cloned wild-type copy of $traR_{pAoF64/95}$ complements the *traR* mutation, it does so only in cultures grown with MOP. This observation suggests that regulation of transfer of pAoF64/95 is more complex than simple transcriptional activation of *traR*.

4.10 Future research will assess how transfer of pAoF64/95 is regulated in a mannopine-dependent manner

Although *traR* is moncistronic and located in the *tra*/*trb* region, which is distal to the MOP catabolism and transport genes, the gene still could be transcriptionally induced by a MOP-responsive regulator. This would allow levels of TraR to titrate the inhibitory effect of TraM. This model is similar to how opines induce transfer of the Ti plasmids and how recipient-produced acyl-HSL induces transfer of pRL1JI. However, the observation that expression of $traR_{pAoF64/95}$ cannot bypass the requirement for MOP implies that regulation of transfer of pAoF64/95 is more complex than simple transcriptional control of *traR*. Considering this, we posit a second model where *traR* is expressed at a low constitutive level, similar to the

expression of *traR* from another Class II plasmid, p42a, and that the availability of MOP somehow modulates the level of TraM, thereby allowing accumulation of sufficient amounts of the transcriptional activator to activate the *tra* regulon. The cells could accomplish this in at least two ways. First, *traM* could be transcriptionally controlled by MOP such that reduced quantities of the antiactivator are made in the presence of the conjugative opine. Alternatively, TraM could be controlled post transcriptionally, possibly by targeted proteolysis or some mechanism that sequesters the antiactivator in cells grown with the conjugative opine. Future research will address each model using gene reporter systems and targeted mutational analysis.

Chapter 5: References

1. **Akiyoshi DE, Morris RO, Hinz R, Mischke BS, Kosuge T, Garfinkel DJ, Gordon MP, Nester EW.** 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc Natl Acad Sci USA **80:**407-411.

2. **Albright LM, Yanofsky MF, Leroux B, Ma DQ, Nester EW.** 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. JBacteriol **169:**1046-1055.

3. **Anderson AR, Moore LW.** 1979. Host specificity in the genus *Agrobacterium*. Phytopathology **69:**320-323.

4. **Bailly X, Giuntini E, Sexton MC, Lower RPJ, Harrison PW, Kumar N, Young JPW.** 2011. Population genomics of *Sinorhizobium medicae* based on low-coverage sequencing of sympatric isolates. ISME J **5:**1722-1734.

5. **Barry GF, Rogers SG, Fraley RT, Brand L.** 1984. Identification of a cloned cytokinin biosynthetic gene. Proc Natl Acad Sci USA **81:**4776-4780.

6. **Bartosik D, Baj J, Wlodarczyk M.** 1998. Molecular and functional analysis of pTAV320, a *repABC*-type replicon of the *Paracoccus versutus* composite plasmid pTAV1. Microbiology **144:**3149-3157.

7. **Bartosik D, Szymanik M, Wysocka E.** 2001. Identification of the partitioning site within the *repABC*-type replicon of the composite *Paracoccus versutus* plasmid pTAV1. J Bacteriol **183:**6234-6243.

8. **Beck von Bodman S, Hayman GT, Farrand SK.** 1992. Opine catabolism and conjugal transfer of the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. Proc Natl Acad Sci USA **89:**643-647.

9. **Beck von Bodman S, Majerczak DR, Coplin DL.** 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. stewartii. Proc Natl Acad Sci USA **95:**7687-7692.

10. **Beck von Bodman S, McCutchan JE, Farrand SK.** 1989. Characterization of conjugal transfer functions of *Agrobacterium tumefaciens* Ti plasmid pTiC58. J Bacteriol **171:**5281-5289.

11. **Beitz E.** 2000. TeXshade: shading and labeling of multiple sequence alignments using LaTeX2e. Bioinformatics **16:**135-139.

12. **Berger BR, Christie PJ.** 1994. Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. J Bacteriol **176:**3646-3660.

13. **Biemann K, Lioret C, Asselineau J, Lederer E, Polonski J.** 1960. Sur la structure chimique de la lysopine, nouvel acide aminé isolé des tissus de crown gall. Bull Soc Chim Fr **42:**979-991.

14. **Bouzar H, Moore LW.** 1987. Complementary methodologies to identify specific *Agrobacterium* strains. Appl Environ Microbiol **53:**2660-2665.

15. **Braun AC.** 1941. Development of secondary tumors and tumor strands in the crown gall of sunflowers. Phytopathology **31:**135-149.

16. **Braun AC.** 1943. Studies on tumor inception in the crown-gall disease. Am J Bot **30:**674-677.

17. **Brevet J, Borowski D, Tempé J.** 1988. Identification of the region encoding opine synthesis and of a region involved in hairy root induction on the T-DNA of cucumber-type Ri plasmid. Mol Plant Microbe Interact **1:**75-79.

18. **Brom S, García-de los Santos A, Cervantes L, Palacios R, Romero D.** 2000. In *Rhizobium etli* symbiotic plasmid transfer, nodulation competitivity and cellular growth require interaction among different replicons. Plasmid **44:**34-43.

19. **Buchmann I, Marner F-J, Schröder G, Waffenschmidt S, Schröder J.** 1985. Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. EMBO J **4:**853-859.

20. **Cangelosi GA, Best EA, Martinetti G, Nester EW.** 1991. Genetic analysis of *Agrobacterium*, *In* Jeffrey HM (ed), Methods in enzymology, vol 204. Academic Press. 384-397.

21. **Castillo-Ramirez S, Vazquez-Castellanos JF, Gonzalez V, Cevallos MA.** 2009. Horizontal gene transfer and diverse functional constraints within a common replication-partitioning system in Alphaproteobacteria: the *repABC* operon. BMC Genomics **10:**536.

22. **Cervantes L, Bustos P, Girard L, Santamaría R, Dávila G, Vinuesa P, Romero D, Brom S.** 2011. The conjugative plasmid of a bean-nodulating *Sinorhizobium fredii* strain is assembled from sequences of two *Rhizobium* plasmids and the chromosome of a *Sinorhizobium* strain. BMC Microbiol **11:**149.

23. **Cervantes-Rivera R, Pedraza-Lopez F, Perez-Segura G, Cevallos MA.** 2011. The replication origin of a *repABC* plasmid. BMC Microbiol **11:**158.

24. **Cevallos MA.** 2002. *Rhizobium etli* CFN42 contains at least three plasmids of the *repABC* family: a structural and evolutionary analysis. Plasmid **48:**104-116.

25. **Cevallos MA, Cervantes-Rivera R, Gutiérrez-Ríos RM.** 2008. The *repABC* plasmid family. Plasmid **60:**19-37.

26. **Chai Y, Winans SC.** 2005. RepB protein of an *Agrobacterium tumefaciens* Ti plasmid binds to two adjacent sites between *repA* and *repB* for plasmid partitioning and autorepression. Mol Microbiol **58:**1114-1129.

27. **Chai Y, Winans SC.** 2005. A small antisense RNA downregulates expression of an essential replicase protein of an *Agrobacterium tumefaciens* Ti plasmid. Mol Microbiol **56:**1574-1585.

28. **Chai Y, Zhu J, Winans SC.** 2001. TrlR, a defective TraR-like protein of *Agrobacterium tumefaciens*, blocks TraR function *in vitro* by forming inactive TrlR:TraR dimers. Mol Microbiol **40:**414-421.

29. **Charles TC, Jin S, Nester EW.** 1992. Two-component sensory transduction systems in phytobacteria. Annu Rev Phytopathol **30:**463-484.

30. **Chen G, Jeffrey PD, Fuqua C, Shi Y, Chen L.** 2007. Structural basis for antiactivation in bacterial quorum sensing. Proc Natl Acad Sci USA **104:**16474-16479.

31. **Chen L, Chen Y, Wood DW, Nester EW.** 2002. A new type IV secretion system promotes conjugal transfer in *Agrobacterium tumefaciens*. J Bacteriol **184:**4838-4845.

32. **Chilton M-D, Drummond MH, Merlo DJ, Sciaky D.** 1978. Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumours. Nature **275:**147-149.

33. **Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW.** 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell **11:**263-271.

34. **Chilton WS, Chilton MD.** 1984. Mannityl opine analogs allow isolation of catabolic pathway regulatory mutants. J Bacteriol **158:**650-658.

35. **Chilton WS, Petit A, Chilton M-D, Dessaux Y.** 2001. Structure and characterization of the crown gall opines heliopine, vitopine and ridéopine. Phytochemistry **58:**137-142.

36. **Chilton WS, Stomp AM, Beringue V, Bouzar H, Vaudequin-Dransart V, Petit A, Dessaux Y.** 1995. The chrysopine family of amadori-type crown gall opines. Phytochemistry **40:**619-628.

37. **Cho H, Pinto UM, Winans SC.** 2009. Transsexuality in the rhizosphere: quorum sensing reversibly converts *Agrobacterium tumefaciens* from phenotypically female to male. J Bacteriol **191:**3375-3383.

38. **Cho H, Winans SC.** 2007. TraA, TraC and TraD autorepress two divergent quorum-regulated promoters near the transfer origin of the Ti plasmid of *Agrobacterium tumefaciens*. Mol Microbiol **63:**1769-1782.

39. **Cho K, Fuqua C, Martin BS, Winans SC.** 1996. Identification of *Agrobacterium tumefaciens* genes that direct the complete catabolism of octopine. J Bacteriol **178:**1872-1880.

40. **Clare BG, Kerr A, Jones DA.** 1990. Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. Plasmid **23:**126-137.

41. **Cook DM, Farrand SK.** 1992. The *oriT* region of the *Agrobacterium tumefaciens* Ti plasmid pTiC58 shares DNA sequence identity with the transfer origins of RSF1010 and RK2/RP4 and with T-region borders. J Bacteriol **174:**6238-6246.

42. **Cook DM, Li PL, Ruchaud F, Padden S, Farrand SK.** 1997. Ti plasmid conjugation is independent of *vir*: reconstitution of the *tra* functions from pTiC58 as a binary system. J Bacteriol **179:**1291-1297.

43. **Costa ED, Chai Y, Winans SC.** 2012. The quorum-sensing protein TraR of *Agrobacterium tumefaciens* is susceptible to intrinsic and TraM-mediated proteolytic instability. Mol Microbiol **84:**807-815.

44. **Danino VE, Wilkinson A, Edwards A, Downie JA.** 2003. Recipient-induced transfer of the symbiotic plasmid pRL1JI in *Rhizobium leguminosarum* bv. *viciae* is regulated by a quorum-sensing relay. Mol Microbiol **50:**511-525.

45. **Darzins A, Chakrabarty AM.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J Bacteriol **159:**9-18.

46. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA **97:**6640-6645.

47. **Davioud E, Petit A, Tate ME, Ryder MH, Tempé J.** 1988. Cucumopine—a new T-DNA-encoded opine in hairy root and crown gall. Phytochemistry **27:**2429-2433.

48. **Davioud E, Quirion J-C, Tate ME, Tempé J, Husson H-P.** 1988. Structure and synthesis of cucumopine, a new crown gall and hairy-root opine. Heterocycles **27:**2423-2429.

49. **De Greve H, Decraemer H, Seurinck J, Van Montagu M, Schell J.** 1981. The functional organization of the octopine *Agrobacterium tumefaciens* plasmid pTiB6S3. Plasmid **6:**235-248.

50. **De Greve H, Dhaese P, Seurinck J, Lemmers M, Van Montagu M, Schell J.** 1982. Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J Mol Appl Genet **1:**499-511.

51. **de la Bastide M, McCombie WR.** 2002. Assembling genomic DNA sequences with PHRAP, Current protocols in bioinformatics doi:10.1002/0471250953.bi1104s17. John Wiley & Sons, Inc.

52. **Delcher AL, Harmon D, Kasif S, White O, Salzberg SL.** 1999. Improved microbial gene identification with GLIMMER. Nucleic Acids Res **27:**4636-4641.

53. **Depicker A, Van Montagu M, Schell J.** 1978. Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity. Nature **275:**150-153.

54. **Dessaux Y, Guyon P, Farrand SK, Petit A, Tempé J.** 1986. *Agrobacterium* Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine. J Gen Microbiol **132:**2549-2559.

55. **Dessaux Y, Petit A, Farrand SK, Murphy PJ.** 1998. Opines and opine-like molecules involved in plant-Rhizobiaceae interactions, *In* Spaink H, Kondorosi A, Hooykaas PJ (ed), The Rhizobiaceae doi:10.1007/978-94-011-5060-6_9. Springer Netherlands. 173-197.

56. **Dessaux Y, Petit A, Tempé J.** 1992. Opines in *Agrobacterium* biology, *In* Verma DPS (ed), Molecular signals in plant-microbe communications. CRC Press, Boca Raton. 109-136.

57. **Dessaux Y, Tempé J, Farrand SK.** 1987. Genetic analysis of mannityl opine catabolism in octopine-type *Agrobacterium tumefaciens* strain 15955. Mol Gen Genet **208:**301-308.

58. **diCenzo G, Milunovic B, Cheng J, Finan TM.** 2013. The $tRNA^{arg}$ gene and *engA* are essential genes on the 1.7-Mb pSymB megaplasmid of *Sinorhizobium meliloti* and were translocated together from the chromosome in an ancestral strain. J Bacteriol **195:**202-212.

59. **Ding H, Hynes MF.** 2009. Plasmid transfer systems in the rhizobia. Can J Microbiol **55:**917-927.

60. **Downie JA, Hombrecher G, Ma Q-S, Knight CD, Wells B, Johnston AWB.** 1983. Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Mol Gen Genet **190:**359-365.

61. **Edgar RC.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113-113.

62. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792-1797.

63. **Eisenbrandt R, Kalkum M, Lai E-M, Lurz R, Kado CI, Lanka E.** 1999. Conjugative pili of IncP plasmids, and the Ti plasmid T pilus are composed of cyclic subunits. J Biol Chem **274:**22548-22555.

64. **Ellis JG, Kerr A, Petit A, Tempé J.** 1982. Conjugal transfer of nopaline and agropine Ti-plasmids - the role of agrocinopines. Mol Gen Genet **186:**269-274.

65. **Ellis JG, Murphy PJ.** 1981. Four new opines from crown gall tumours- their detection and properties. Mol Gen Genet **181:**36-43.

66. **Ellis JG, Murphy PJ, Kerr A.** 1982. Isolation and properties of transfer regulatory mutants of the nopaline Ti-plasmid pTiC58. Mol Gen Genet **186:**275-281.

67. **Ellis JG, Ryder MH, Tate ME.** 1984. *Agrobacterium tumefaciens* TR-DNA encodes a pathway for agropine biosynthesis. Mol Gen Genet **195:**466-473.

68. **Engler G, Depicker A, Maenhaut R, Villarroel R, Van Montagu M, Schell J.** 1981. Physical mapping of DNA base sequence homologies between an octopine and a nopaline Ti plasmid of *Agrobacterium tumefaciens*. J Mol Biol **152:**183-208.

69. **Ewing B, Green P.** 1998. Base-calling of automated sequencer traces using *Phred.* II. error probabilities. Genome Res **8:**186-194.

70. **Ewing B, Hillier L, Wendl MC, Green P.** 1998. Base-calling of automated sequencer traces using *Phred.* I. accuracy assessment. Genome Res **8:**175-185.

71. **Farrand S, Hwang I, Cook D.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J Bacteriol **178:**4233-4247.

72. **Farrand SK.** 1998. Conjugal plasmids and their transfer, *In* Spaink H, Kondorosi A, Hooykaas PJ (ed), The Rhizobiaceae doi:10.1007/978-94-011-5060-6_10. Springer Netherlands. 199-233.

73. **Farrand SK, Hwang I, Cook DM.** 1996. The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. J Bacteriol **178:**4233-4247.

74. **Farrand SK, Qin Y, Oger P.** 2002. Quorum-sensing system of *Agrobacterium* plasmids: analysis and utility, *In* Virginia L. Clark PMB (ed), Methods in enzymology, vol 358. Academic Press. 452-484.

75. **Farrand SK, Slota JE, Shim JS, Kerr A.** 1985. Tn5 insertions in the agrocin 84 plasmid: the conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. Plasmid **13:**106-117.

76. **Farrand SK, Tempé J, Dessaux Y.** 1990. Localization and characterization of the region encoding catabolism of mannopinic acid from the octopine-type Ti plasmid pTi15955. Mol Plant Microbe Interact **3:**259-267.

77. **Felsenstein J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783-791.

78. **Firmin JL, Fenwick GR.** 1978. Agropine- a major new plasmid-determined metabolite in crown gall tumours. Nature **276:**842-844.

79. **Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394-401.

80. **Fullner KJ.** 1998. Role of *Agrobacterium virB* genes in transfer of T complexes and RSF1010. J Bacteriol **180:**430-434.

81. **Fuqua C, Burbea M, Winans S.** 1995. Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the *traM* gene. J Bacteriol **177:**1367-1373.

82. **Fuqua C, Winans SC.** 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J Bacteriol **176:**2796-2806.

83. **Fuqua C, Winans SC.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J Bacteriol **178:**435-440.

84. **Fuqua C, Winans SC.** 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the *traR* gene of Ti plasmid pTiR10. Mol Microbiol **20:**1199-1210.

85. **Fuqua WC, Winans SC, Greenberg EP.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol **176:**269-275.

86. **Galibert F, Finan TM, Long SR, Pühler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis RW, Dréano S, Federspiel NA, Fisher RF, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman RW, Jones T, Kahn D, Kahn ML, Kalman S, Keating DH, Kiss E, Komp C, Lelaure V, Masuy D, Palm C, Peck MC, Pohl TM, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thébault P, Vandenbol M, et al.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668-672.

87. **Gelvin SB.** 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol **51:**223-256.

88. **Genetello C, Van Larebeke N, Holsters M, De Picker A, Van Montagu M, Schell J.** 1977. Ti plasmids of *Agrobacterium* as conjugative plasmids. Nature **265:**561-563.

89. **Giusti MdlÁ, Pistorio M, Lozano MJ, Torres Tejerizo GA, Salas ME, Martini MC, López JL, Draghi WO, Del Papa MF, Pérez-Mendoza D, Sanjuán J, Lagares A.** 2012. Genetic and functional characterization of a yet-unclassified rhizobial Dtr (DNA-transfer-and-replication) region from a ubiquitous plasmid conjugal system present in *Sinorhizobium meliloti*, in *Sinorhizobium medicae*, and in other nonrhizobial Gram-negative bacteria. Plasmid **67:**199-210.

90. **Goldman A, Tempé J, Morel G.** 1968. Quelques particularities de diverse souches d'*Agrobacterium tumefaciens*. CR Soc Biol (Paris) **162:**630-631.

91. **Goldmann A, Thomas DW, Morel G.** 1969. Sur la structure de la nopaline, métabolite anormal de certaines tumeurs de crown gall. CR Acad Sci Ser D **268:**852-854.

92. **González V, Bustos P, Ramírez-Romero MA, Medrano-Soto A, Salgado H, Hernández-González I, Hernández-Celis JC, Quintero V, Moreno-Hagelsieb G, Girard L, Rodríguez O, Flores M, Cevallos MA, Collado-Vides J, Romero D, Dávila G.** 2003. The mosaic structure of the symbiotic plasmid of *Rhizobium etli* CFN42 and its relation to other symbiotic genome compartments. Genome Biol **4:**R36.

93. **González V, Santamaría RI, Bustos P, Hernández-González I, Medrano-Soto A, Moreno-Hagelsieb G, Janga SC, Ramírez MA, Jiménez-Jacinto V, Collado-Vides J, Dávila G.** 2006. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc Natl Acad Sci USA **103:**3834-3839.

94. **Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A, Liu F, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C, Crowell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C, Slater S.** 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. Science **294:**2323-2328.

95. **Green P.** 1996. Documentation for PHRAP, *on* Genome Center, University of Washington. http://bozeman.mbt.washington.edu.

96. **Haase J, Lanka E.** 1997. A specific protease encoded by the conjugative DNA transfer systems of IncP and Ti plasmids is essential for pilus synthesis. J Bacteriol **179:**5728-5735.

97. **Habeeb LF, Wang L, Winans SC.** 1991. Transcription of the octopine catabolism operon of the *Agrobacterium* tumor-inducing plasmid pTiA6 is activated by a LysR-type regulatory protein. Mol Plant Microbe Interact **4:**379-385.

98. **Hamilton CM, Lee H, Li P-L, Cook DM, Piper KR, von Bodman SB, Lanka E, Ream W, Farrand SK.** 2000. TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. J Bacteriol **182:**1541-1548.

99. **Hamilton RH, Fall MZ.** 1971. The loss of tumor-initiating ability in *Agrobacterium tumefaciens* by incubation at high temperature. Experientia **27:**229-230.

100. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557-580.

101. **Harrison PW, Lower RPJ, Kim NKD, Young JPW.** 2010. Introducing the bacterial 'chromid': not a chromosome, not a plasmid. Trends Microbiol **18:**141-148.

102. **Hayman GT, Farrand SK.** 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. J Bacteriol **170:**1759-1767.

103. **Hayman GT, Farrand SK.** 1990. *Agrobacterium* plasmids encode structurally and functionally different loci for catabolism of agrocinopine-type opines. Mol Gen Genet **223:**465-473.

104. **He X, Chang W, Pierce DL, Seib LO, Wagner J, Fuqua C.** 2003. Quorum sensing in *Rhizobium sp*. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. J Bacteriol **185:**809-822.

105. **Herrera-Cervera JA, Sanjuan-Pinilla JM, Olivares J, Sanjuan J.** 1998. Cloning and identification of conjugative transfer origins in the *Rhizobium meliloti* genome. J Bacteriol **180:**4583-4590.

106. **Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, Schell J.** 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet **163:**181-187.

107. **Holsters M, Silva B, Van Vliet F, Genetello C, De Block M, Dhaese P, Depicker A, Inzé D, Engler G, Villarroel R, Van Montagu M, Schell J.** 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3:**212-230.

108. **Hong S, Farrand S.** 1996. Purification and characterization of catabolic mannopine cyclase encoded by the *Agrobacterium tumefaciens* Ti plasmid pTi15955. J Bacteriol **178:**2427-2430.

109. **Hong SB, Dessaux Y, Chilton WS, Farrand SK.** 1993. Organization and regulation of the mannopine cyclase-associated opine catabolism genes in *Agrobacterium tumefaciens* 15955. J Bacteriol **175:**401-410.

110. **Hong SB, Farrand SK.** 1994. Functional role of the Ti plasmid-encoded catabolic mannopine cyclase in mannityl opine catabolism by *Agrobacterium spp*. J Bacteriol **176:**3576-3583.

111. **Hooykaas PJ, den Dulk-Ras H, Ooms G, Schilperoort RA.** 1980. Interactions between octopine and nopaline plasmids in *Agrobacterium tumefaciens*. J Bacteriol **143:**1295-1306.

112. **Hooykaas PJJ, Beijersbergen AGM.** 1994. The virulence system of *Agrobacterium tumefaciens*. Annu Rev Phytopathol **32:**157-181.

113. **Huffman GA, White FF, Gordon MP, Nester EW.** 1984. Hairy-root-inducing plasmid: physical map and homology to tumor-inducing plasmids. J Bacteriol **157:**269-276.

114. **Hwang I, Cook D, Farrand S.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J Bacteriol **177:**449-458.

115. **Hwang I, Li PL, Zhang L, Piper KR, Cook DM, Tate ME, Farrand SK.** 1994. TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer. Proc Natl Acad Sci USA **91:**4639-4643.

116. **Hwang I, Smyth AJ, Luo Z-Q, Farrand SK.** 1999. Modulating quorum sensing by antiactivation: TraM interacts with TraR to inhibit activation of Ti plasmid conjugal transfer genes. Mol Microbiol **34:**282-294.

117. **Inzé D, Follin A, Lijsebettens M, Simoens C, Genetello C, Montagu M, Schell J.** 1984. Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. Mol Gen Genet **194:**265-274.

118. **Isogai A, Fukuchi N, Hayashi M, Kamada H, Harada H, Suzuki A.** 1988. Structure of a new opine, mikimopine, in hairy root induced by *Agrobacterium rhizogenes*. Agric Biol Chem **52:**3235-3237.

119. **Isogai A, Fukuchi N, Hayashi M, Kamada H, Harada H, Suzuki A.** 1990. Mikimopine, an opine in hairy roots of tobacco induced by *Agrobacterium rhizogenes*. Phytochemistry **29:**3131-3134.

120. **Jayaswal RK, Veluthambi K, Gelvin SB, Slightom JL.** 1987. Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in Escherichia coli by a virD-encoded border-specific endonuclease from Agrobacterium tumefaciens. Journal of Bacteriology **169:**5035-5045.

121. **Joos H, Inzé D, Caplan A, Sormann M, Van Montagu M, Schell J.** 1983. Genetic analysis of T-DNA transcripts in nopaline crown galls. Cell **32:**1057-1067.

122. **Jouanin L, Tourneur J, Tourneur C, Casse-Delbart F.** 1986. Restriction maps and homologies of the three plasmids of *Agrobacterium rhizogenes* strain A4. Plasmid **16:**124-134.

123. **Jung W-H, Baek C-H, Lee J-K, Kim K-S.** 1999. Analysis of trans-acting elements for regulation of *moc* operons of pTi15955 in *Agrobacterium tumefaciens*. J Microbiol Biotechnol **9:**637-645.

124. **Kalogeraki VS, Winans SC.** 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. Gene **188:**69-75.

125. **Katoh K, Toh H.** 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform **9:**286-298.

126. **Keane PJ, Kerr A, New PB.** 1970. Crown gall of stone fruit II. identification and nomenclature of *Agrobacterium* isolates. Aust J Biol Sci **23:**585-596.

127. **Kerr A.** 1969. Crown gall of stone fruit I. isolation of *Agrobacterium tumefaciens* and related species. Aust J Biol Sci **22:**111-116.

128. **Kerr A.** 1969. Transfer of virulence between isolates of *Agrobacterium*. Nature **223:**1175-1176.

129. **Kerr A.** 1971. Acquisition of virulence by non-pathogenic isolates of *Agrobacterium radiobacter*. Physiol Plant Path **1:**241-246.

130. **Kerr A, Manigault P, Tempé J.** 1977. Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature **265:**560-561.

131. **Khan SR, Gaines J, Roop RM, Farrand SK.** 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. App Environ Microbiol **74:**5053-5062.

132. **Kim H, Farrand S.** 1997. Characterization of the *acc* operon from the nopaline-type Ti plasmid pTiC58, which encodes utilization of agrocinopines A and B and susceptibility to agrocin 84. J Bacteriol **179:**7559-7572.

133. **Kim K, Chilton W, Farrand S.** 1996. A Ti plasmid-encoded enzyme required for degradation of mannopine is functionally homologous to the T-region-encoded enzyme required for synthesis of this opine in crown gall tumors. J Bacteriol **178:**3285-3292.

134. **Kim K, Farrand S.** 1996. Ti plasmid-encoded genes responsible for catabolism of the crown gall opine mannopine by *Agrobacterium tumefaciens* are homologs of the T- region genes responsible for synthesis of this opine by the plant tumor. J Bacteriol **178:**3275-3284.

135. **Kim S-I, Veena, Gelvin SB.** 2007. Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. Plant J **51:**779-791.

136. **Kiyokawa S, Kobayashi K, Kikuchi Y, Kamada H, Harada H.** 1994. Root-inducing region of mikimopine type Ri plasmid pRi1724. Plant Physiol **104:**801-802.

137. **Koekman BP, Ooms G, Klapwijk PM, Schilperoort RA.** 1979. Genetic map of an octopine TI-plasmid. Plasmid **2:**347-357.

138. **Koukolíková-Nicola Z, Raineri D, Stephens K, Ramos C, Tinland B, Nester EW, Hohn B.** 1993. Genetic analysis of the *virD* operon of *Agrobacterium tumefaciens*: a search for functions involved in transport of T-DNA into the plant cell nucleus and in T-DNA integration. J Bacteriol **175:**723-731.

139. **Landeta C, Dávalos A, Cevallos MÁ, Geiger O, Brom S, Romero D.** 2011. Plasmids with a chromosome-like role in rhizobia. J Bacteriol **193:**1317-1326.

140. **Lessl M, Pansegrau W, Lanka E.** 1992. Relationship of DNA-transfer-systems: essential transfer factors of plasmids RP4, Ti and F share common sequences. Nucleic Acids Res **20:**6099-6100.

141. **Li PL.** 2000. Molecular genetic characterization of the mating pair formation and vegitative replication regions on the *Agrobacterium tumefaciens* Ti plasmid pTiC58. PhD. University of Illinois.

142. **Li PL, Everhart DM, Farrand SK.** 1998. Genetic and sequence analysis of the pTiC58 *trb* locus, encoding a mating-pair formation system related to members of the type IV secretion family. J Bacteriol **180:**6164-6172.

143. **Li PL, Farrand SK.** 2000. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. J Bacteriol **182:**179-188.

144. **Li PL, Hwang I, Miyagi H, True H, Farrand SK.** 1999. Essential components of the Ti plasmid *trb* system, a type IV macromolecular transporter. J Bacteriol **181:**5033-5041.

145. **Lin T-S, Kado CI.** 1993. The *virD4* gene is required for virulence while *virD3* and *orf5* are not required for virulence of *Agrobacterium tumefaciens*. Mol Microbiol **9:**803-812.

146. **Lioret C.** 1956. Sur la mise en évidence d'un acide aminé non identifié particulier aux tissus de crown gall. Bull Soc Fr Physiol Vég **2:**76.

147. **Lioret C.** 1957. Les acides aminés libres des tissus de crown gall. Mise en évidence d'un acide aminé particulier ces tissus. CR Acad Sci **244:**2171.

148. **Lippincott JA, Beiderbeck R, Lippincott BB.** 1973. Utilization of octopine and nopaline by *Agrobacterium*. J Bacteriol **116:**378-383.

149. **Lithgow JK, Wilkinson A, Hardman A, Rodelas B, Wisniewski-Dyé F, Williams P, Downie JA.** 2000. The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. Mol Microbiol **37:**81-97.

150. **Luo ZQ, Clemente TE, Farrand SK.** 2001. Construction of a derivative of *Agrobacterium tumefaciens* C58 that does not mutate to tetracycline resistance. Mol Plant Microbe Interact **14:**98-103.

151. **Luo ZQ, Farrand SK.** 1999. Signal-dependent DNA binding and functional domains of the quorum-sensing activator TraR as identified by repressor activity. Proc Natl Acad Sci USA **96:**9009-9014.

152. **Luo ZQ, Qin Y, Farrand SK.** 2000. The antiactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. J Biol Chem **275:**7713-7722.

153. **Luo ZQ, Smyth AJ, Gao P, Qin Y, Farrand SK.** 2003. Mutational analysis of TraR: correlating function with molecular structure of a quorum-sensing transcriptional activator. J Biol Chem **278:**13173-13182.

154. **Luo ZQ, Su S, Farrand SK.** 2003. *In situ* activation of the quorum-sensing transcription factor TraR by cognate and noncognate acyl-homoserine lactone ligands: kinetics and consequences. J Bacteriol **185:**5665-5672.

155. **Lyi SM, Jafri S, Winans SC.** 1999. Mannopinic acid and agropinic acid catabolism region of the octopine-type Ti plasmid pTi15955. Mol Microbiol **31:**339-347.

156. **MacLellan SR, Smallbone LA, Sibley CD, Finan TM.** 2005. The expression of a novel antisense gene mediates incompatibility within the large *repABC* family of alpha-proteobacterial plasmids. Mol Microbiol **55:**611-623.

157. **MacLellan SR, Zaheer R, Sartor AL, MacLean AM, Finan TM.** 2006. Identification of a megaplasmid centromere reveals genetic structural diversity within the *repABC* family of basic replicons. Mol Microbiol **59:**1559-1575.

158. **Mankin SL, Hill DS, Olhoft PM, Toren E, Wenck AR, Nea L, Xing L, Brown JA, Fu H, Ireland L, Jia H, Hillebrand H, Jones T, Song H-S, Ozias-Akins P.** 2007. Disarming and sequencing of *Agrobacterium rhizogenes* strain K599 (NCPPB2659) plasmid pRi2659. *In Vitro* Cell Dev Biol, Plant **43:**521-535.

159. **Mavingui P, Flores M, Guo X, Dávila G, Perret X, Broughton WJ, Palacios R.** 2002. Dynamics of genome architecture in *Rhizobium* sp. strain NGR234. J Bacteriol **184:**171-176.

160. **McAnulla C, Edwards A, Sanchez-Contreras M, Sawers RG, Downie JA.** 2007. Quorum-sensing-regulated transcriptional initiation of plasmid transfer and replication genes in *Rhizobium leguminosarum* biovar *viciae*. Microbiology **153:**2074-2082.

161. **Ménagé A, Morel G.** 1964. Sur la présence d'octopine dans les tissus de crown gall. CR Acad Sci **259:**4795-4796.

162. **Ménagé A, Morel G.** 1965. Sur la présence d'un acide aminè nouveau dans le tissu de crown-gall. CR Soc Biol **159:**561-562.

163. **Merlo DJ, Nester EW.** 1977. Plasmids in avirulent strains of *Agrobacterium*. J Bacteriol **129:**76-80.

164. **Metcalf WW, Jiang W, Daniels LL, Kim S-K, Haldimann A, Wanner BL.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZα* for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35:**1-13.

165. **Minogue TD, Wehland von Trebra M, Bernhard F, Beck von Bodman S.** 2002. The autoregulatory role of EsaR, a quorum-sensing regulator in *Pantoea stewartii* ssp. *stewartii*: evidence for a repressor function. Mol Microbiol **44:**1625-1635.

166. **Montoya AL, Moore LW, Gordon MP, Nester EW.** 1978. Multiple genes coding for octopine-degrading enzymes in *Agrobacterium*. J Bacteriol **136:**909-915.

167. **Moore L, Warren G, Strobel G.** 1979. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid **2:**617-626.

168. **Moore LW, Chilton WS, Canfield ML.** 1997. Diversity of opines and opine-catabolizing bacteria isolated from naturally occurring crown gall tumors. Appl Environ Microbiol **63:**201-207.

169. **Moore LW, Kado CI, Bouzar H.** 1988. *Agrobacterium*, *In* Schaad NW (ed), Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. APS Press, St. Paul. 16-36.

170. **Morel G.** 1956. Métabolisme de l'arginine par les tissus de crown gall de topinambour. Bull Soc Fr Physiol Vég **2:**75.

171. **Moriguchi K, Maeda Y, Satou M, Hardayani NSN, Kataoka M, Tanaka N, Yoshida K.** 2001. The complete nucleotide sequence of a plant root-inducing (Ri) plasmid indicates its chimeric structure and evolutionary relationship between tumor-inducing (Ti) and symbiotic (Sym) plasmids in Rhizobiaceae. J Mol Biol **307:**771-784.

172. **Murphy PJ, Heycke N, Banfalvi Z, Tate ME, de Bruijn F, Kondorosi A, Tempé J, Schell J.** 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc Natl Acad Sci USA **84:**493-497.

173. **Murphy PJ, Heycke N, Trenz SP, Ratet P, de Bruijn FJ, Schell J.** 1988. Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc Natl Acad Sci USA **85:**9133-9137.

174. **Nei M, Kumar S.** 2000. Molecular evolution and phylogenetics. Oxford University Press.

175. **Nesme X, Michel M-F, Digat B.** 1987. Population heterogeneity of *Agrobacterium tumefaciens* in galls of *Populus* L. from a single nursery. Appl Environ Microbiol **53:**655-659.

176. **Novick RP, Hoppensteadt FC.** 1978. On plasmid incompatibility. Plasmid **1:**421-434.

177. **Oger P, Farrand SK.** 2001. Co-evolution of the agrocinopine opines and the agrocinopine-mediated control of TraR, the quorum-sensing activator of the Ti plasmid conjugation system. Mol Microbiol **41:**1173-1185.

178. **Oger P, Farrand SK.** 2002. Two opines control conjugal transfer of an *Agrobacterium* plasmid by regulating expression of separate copies of the quorum-sensing activator gene *traR*. J Bacteriol **184:**1121-1131.

179. **Oger P, Kim K-S, Sackett RL, Piper KR, Farrand SK.** 1998. Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. Mol Microbiol **27:**277-288.

180. **Palanichelvam K, Oger P, Clough SJ, Cha C, Bent AF, Farrand SK.** 2000. A second T-region of the soybean-supervirulent chrysopine-type Ti plasmid pTiChry5, and construction of a fully disarmed *vir* helper plasmid. Mol Plant Microbe Interact **13:**1081-1091.

181. **Pappas KM, Winans SC.** 2003. A LuxR-type regulator from *Agrobacterium tumefaciens* elevates Ti plasmid copy number by activating transcription of plasmid replication genes. Mol Microbiol **48:**1059-1073.

182. **Paulus F, Otten L.** 1993. Functional and mutated agrocinopine synthase genes on octopine T-DNAs. Mol Plant Microbe Interact **6:**393-402.

183. **Pérez-Mendoza D, Sepúlveda E, Pando V, Muñoz S, Nogales J, Olivares J, Soto MJ, Herrera-Cervera JA, Romero D, Brom S, Sanjuán J.** 2005. Identification of the *rctA* gene, which is required for repression of conjugative transfer of rhizobial symbiotic megaplasmids. J Bacteriol **187:**7341-7350.

184. **Pérez-Oseguera Á, Cevallos MA.** 2013. RepA and RepB exert plasmid incompatibility repressing the transcription of the *repABC* operon. Plasmid **70:**362-376.

185. **Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempé J.** 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol Gen Genet **190:**204-214.

186. **Petit A, Delhaye S, Tempé J, Morel G.** 1970. Recherches sur les guanidines des tissus de crown gall. Mise en évidence d'une relation biochimique specifique entre les souches d'*Agrobacterium tumefaciens* et les tumeurs qu'elles induisent. Physiol vég **8:**205-213.

187. **Petit A, Tempé J, Kerr A, Holsters M, Van Montagu M, Schell J.** 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature **271:**570-572.

188. **Pinto UM, Pappas KM, Winans SC.** 2012. The ABCs of plasmid replication and segregation. Nat Rev Micro **10:**755-765.

189. **Piper KR, Beck von Bodman S, Farrand SK.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448-450.

190. **Piper KR, Beck von Bodman S, Hwang I, Farrand SK.** 1999. Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium*. Mol Microbiol **32:**1077-1089.

191. **Piper KR, Farrand SK.** 2000. Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. J Bacteriol **182:**1080-1088.

192. **Pohlman RF, Genetti HD, Winans SC.** 1994. Common ancestry between IncN conjugal transfer genes and macromolecular export systems of plant and animal pathogens. Mol Microbiol **14:**655-668.

193. **Porter SG, Yanofsky MF, Nester EW.** 1987. Molecular characterization of the *virD* operon from *Agrobacterium tumefaciens*. Nucleic Acids Res **15:**7503-7517.

194. **Qin Y, Luo ZQ, Smyth AJ, Gao P, von Bodman SB, Farrand SK.** 2000. Quorum‐sensing signal binding results in dimerization of TraR and its release from membranes into the cytoplasm. EMBO J **19:**5212-5221.

195. **Ramírez-Romero MA, Soberón N, Pérez-Oseguera A, Téllez-Sosa J, Cevallos MA.** 2000. Structural elements required for replication and incompatibility of the *Rhizobium etli* symbiotic plasmid. J Bacteriol **182:**3117-3124.

196. **Rasband WS.** 1997-2012. ImageJ, National Institutes of Health, Bethesda. http://imagej.nih.gov/ij/.

197. **Ryder MH, Tate ME, Jones GP.** 1984. Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose. J Biol Chem **259:**9704-9710.

198. **Rzhetsky A, Nei M.** 1992. A simple method for estimating and testing Minimum-Evolution trees. Mol Biol Evol **9:**945.

199. **Saint CP, Wexler M, Murphy PJ, Tempé J, Tate ME, Murphy PJ.** 1993. Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. J Bacteriol **175:**5205-5215.

200. **Saitou N, Nei M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406-425.

201. **Salomon F, Deblaere R, Leemans J, Hernalsteens JP, Van Montagu M, Schell J.** 1984. Genetic identification of functions of TR-DNA transcripts in octopine crown galls. EMBO J **3:**141-146.

202. **Salzberg SL, Delcher AL, Kasif S, White O.** 1998. Microbial gene identification using interpolated Markov models. Nucleic Acids Res **26:**544-548.

203. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd Edition ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

204. **Schell J, Van Montagu M, De Beuckeleer M, De Block M, Depicker A, De Wilde M, Engler G, Genetello C, Hernalsteens JP, Holsters M, Seurinck J, Silva B, Van Vliet F, Villarroel R.** 1979. Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host. Proc R Soc Lond [Biol] **204:**251-266.

205. **Schindler U, Sans N, Schröder J.** 1989. Ornithine cyclodeaminase from octopine Ti plasmid Ach5: identification, DNA sequence, enzyme properties, and comparison with gene and enzyme from nopaline Ti plasmid C58. J Bacteriol **171:**847-854.

206. **Schröder G, Waffenschmidt S, Weiler EW, Schröder J.** 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur J Biochem **138:**387-391.

207. **Schröder J, Schröder G, Huisman H, Schilperoort RA, Schell J.** 1981. The mRNA for lysopine dehydrogenase in plant tumor cells is complementary to a Ti-plasmid fragment. FEBS Lett **129:**166-168.

208. **Sepúlveda E, Pérez-Mendoza D, Ramírez-Romero MA, Soto MJ, López-Lara IM, Geiger O, Sanjuán J, Brom S, Romero D.** 2008. Transcriptional Interference and repression modulate the conjugative ability of the symbiotic plasmid of *Rhizobium etli*. J Bacteriol **190:**4189-4197.

209. **Shaw PD, Ping G, Daly SL, Cha C, Cronan JE, Rinehart KL, Farrand SK.** 1997. Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc Natl Acad Sci USA **94:**6036-6041.

210. **Shiomi T, Shirakata T, Takeuchi A, Oizumi T, Uematsu S.** 1987. Hairy root of melon caused by *Agrobacterium rhizogenes* biovar 1. Ann Phytopath Soc Jpn **53:**454-549.

211. **Shirasu K, Kado CI.** 1993. The *virB* operon of the Agrobacterium tumefaciens virulence regulon has sequence similarities to B, Cand D open reading frames downstream of the pertussis toxin-operon and to the DNA transfer-operons of broad-host-range conjugative plasmids. Nucleic Acids Res **21:**353-354.

212. **Simon R, Priefer U, Pühler A.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol **1:**784-791.

213. **Slater SC, Goldman BS, Goodner B, Setubal JC, Farrand SK, Nester EW, Burr TJ, Banta L, Dickerman AW, Paulsen I, Otten L, Suen G, Welch R, Almeida NF, Arnold F, Burton OT, Du Z, Ewing A, Godsy E, Heisel S, Houmiel KL, Jhaveri J, Lu J, Miller NM, Norton S, Chen Q, Phoolcharoen W, Ohlin V, Ondrusek D, Pride N, Stricklin SL, Sun J, Wheeler C, Wilson L, Zhu H, Wood DW.** 2009. Genome sequences of three *Agrobacterium* biovars help elucidate the evolution of multichromosome genomes in bacteria. J Bacteriol **191:**2501-2511.

214. **Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D.** 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J Biol Chem **261:**108-121.

215. **Smith EF, Townsend CO.** 1907. A plant-tumor of bacterial origin. Science **25:**671-673.

216. **Sneath PHA, Sokal RR.** 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman.

217. **Stachel SE, Timmerman B, Zambryski P.** 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature **322:**706-712.

218. **Stonier T.** 1956. Labeling crown gall bacteria with P^{32} for radioautography. J Bacteriol **72:**259-268.

219. **Streit WR, Schmitz RA, Perret X, Staehelin C, Deakin WJ, Raasch C, Liesegang H, Broughton WJ.** 2004. An evolutionary hot spot: the pNGR234b replicon of *Rhizobium* sp. strain NGR234. J Bacteriol **186:**535-542.

220. **Su S, Khan SR, Farrand SK.** 2008. Induction and loss of Ti plasmid conjugative competence in response to the acyl-homoserine lactone quorum-sensing signal. J Bacteriol **190:**4398-4407.

221. **Subramaniam S.** 1998. The biology workbench—A seamless database and analysis environment for the biologist. Proteins: Structure, Function, and Bioinformatics **32:**1-2.

222. **Suzuki K, Hattori Y, Uraji M, Ohta N, Iwata K, Murata K, Kato A, Yoshida K.** 2000. Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. Gene **242:**331-336.

223. **Szegedi E, Czakó M, Otten L.** 1996. Further evidence that the vitopine-type pTi's of *Agrobacterium vitis* represent a novel group of Ti plasmids. Mol Plant Microbe Interact **9:**139-143.

224. **Szegedi E, Otten L.** 1998. Incompatibility properties of tartrate utilization plasmids derived from *Agrobacterium vitis* strains. Plasmid **39:**35-40.

225. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol **30:**2725-2729.

226. **Tate ME, Ellis JG, Kerr A, Tempé J, Murray KE, Shaw KJ.** 1982. Agropine: A revised structure. Carbohydr Res **104:**105-120.

227. **Tate ME, Murphy PJ, Roberts WP, Kerr A.** 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature **280:**697-699.

228. **Tempé J, Guyon P, Petit A, Ellis JG, Tate ME, Kerr A.** 1980. Préparation et propriétés de nouveaux substrats cataboliques pour deux types de plasmides oncognes d'*Agrobacterium tumefaciens*. CR Acad Sci Ser D **290:**1173-1176.

229. **Tempé J, Guyon P, Tepfer D, Petit A.** 1979. The role of opines in the ecology of the Ti-plasmids of *Agrobacterium*, *In* Timmins KN, Pühler A (ed), Plasmids of medical, environmental, and commercial importance. Elsevier/North Holland Biomedical Press, Amsterdam. 353-363.

230. **Tempé J, Petit A.** 1983. La piste des opines, Molecular genetics of the bacteria-plant interaction. Springer-Verlag, New York. 14-32.

231. **Tempé J, Petit A, Bannerot H.** 1982. Presence de substances semblables a des opines dans des nodosites de Luzerne. CR Acad Sci Ser III **295:**413-416.

232. **Tempé J, Petit A, Farrand S.** 1984. Induction of cell proliferation by *Agrobacterium tumefaciens* and *A. rhizogenes*: a parasite's point of view, *In* Verma DPS, Hohn T (ed), Genes involved in microbe-plant interactions doi:10.1007/978-3-7091-8739-5_10. Springer Vienna. 271-286.

233. **Tempé J, Petit A, Holsters M, Montagu Mv, Schell J.** 1977. Thermosensitive step associated with transfer of the Ti plasmid during conjugation: possible relation to transformation in crown gall. Proc Natl Acad Sci USA **74:**2848-2849.

234. **Tepfer DA, Tempé J.** 1981. Production d' agropine par des racines formées sous l' action d' *Agrobacterium rhizogenes*, souche A4. CR Acad Sci Ser III **292:**153-156.

235. **Thomashow LS, Reeves S, Thomashow MF.** 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc Natl Acad Sci USA **81:**5071-5075.

236. **Thomashow MF, Hugly S, Buchholz WG, Thomashow LS.** 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. Science **231:**616-618.

237. **Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW.** 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell **19:**729-739.

238. **Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:**4673-4680.

239. **Tinland B, Hohn B, Puchta H.** 1994. *Agrobacterium tumefaciens* transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus. Proc Natl Acad Sci USA **91:**8000-8004.

240. **Torres Tejerizo G, Pistorio M, Althabegoiti MJ, Cervantes L, Wibberg D, Schlüter A, Pühler A, Lagares A, Romero D, Brom S.** 2014. Rhizobial plasmid pLPU83a is able to switch between different transfer machineries depending on its genomic background. FEMS Microbiol Ecol **88:**565-578.

241. **Trevelyan WE, Procter DP, Harrison JS.** 1950. Detection of sugars on paper chromatograms. Nature **166:**444-445.

242. **Tsai CS, Winans SC.** 2011. The quorum-hindered transcription factor YenR of *Yersinia enterocolitica* inhibits pheromone production and promotes motility via a small non-coding RNA. Mol Microbiol **80:**556-571.

243. **Tun-Garrido C, Bustos P, Gonzalez V, Brom S.** 2003. Conjugative transfer of p42a from *Rhizobium etli* CFN42, which is required for mobilization of the symbiotic plasmid, is regulated by quorum sensing. J Bacteriol **185:**1681-1692.

244. **Valdivia RH, Wang L, Winans SC.** 1991. Characterization of a putative periplasmic transport system for octopine accumulation encoded by *Agrobacterium tumefaciens* Ti plasmid pTiA6. J Bacteriol **173:**6398-6405.

245. **Van den Eede G, Deblaere R, Goethals K, Van Montagu M, Holster M.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol Plant Microbe Interact **5:**228-234.

246. **Van Larebeke N, Engler G, Holsters M, Van den Elsacker S, Zaenen I, Schilperoort RA, Schell J.** 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature **252:**169-170.

247. **Van Onckelen H, Prinsen E, Inzé D, Rüdeisheim P, Van Lijsebettens M, Follin A, Schell J, Van Montagu M, De Greef J.** 1986. *Agrobacterium* T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells. FEBS Letters **198:**357-360.

248. **Vannini A, Volpari C, Gargioli C, Muraglia E, Cortese R, De Francesco R, Neddermann P, Di Marco S.** 2002. The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA, vol 21. 4393-4401.

249. **Vaudequin-Dransart V, Petit A, Poncet C, Ponsonnet C, Nesme X, Jones JB, Bouzar H, Chilton WS, Dessaux Y.** 1995. Novel Ti plasmids in *Agrobacterium* strains isolated from fig tree and chrysanthemum tumors and their opinelike molecules. Mol Plant Microbe Interact **8:**311-321.

250. **Venkova-Canova T, Soberón NE, Ramírez-Romero MA, Cevallos MA.** 2004. Two discrete elements are required for the replication of a *repABC* plasmid: an antisense RNA and a stem-loop structure. Mol Microbiol **54:**1431-1444.

251. **Watson B, Currier TC, Gordon MP, Chilton MD, Nester EW.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J Bacteriol **123:**255-264.

252. **Wetzel ME, Kim KS, Miller M, Olsen GJ, Farrand SK.** 2014. Quorum-dependent mannopine-inducible conjugative transfer of an *Agrobacterium* opine-catabolic plasmid. J Bacteriol **196:**1031-1044.

253. **White CE, Winans SC.** 2007. Cell-cell communication in the plant pathogen *Agrobacterium tumefaciens*. Philos Trans R Soc Lond, B, Biol Sci **362:**1135-1148.

254. **White FF, Nester EW.** 1980. Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J Bacteriol **141:**1134-1141.

255. **White FF, Nester EW.** 1980. Relationship of plasmids responsible for hairy root and crown gall tumorigenicity. J Bacteriol **144:**710-720.

256. **White PR, Braun AC.** 1942. A cancerous neoplasm of plants. Autonomous bacteria-free crown-gall tissue. Cancer Res **2:**597-617.

257. **Wilkinson A, Danino V, Wisniewski-Dye F, Lithgow JK, Downie JA.** 2002. N-acyl-homoserine lactone inhibition of rhizobial growth is mediated by two quorum-sensing genes that regulate plasmid transfer. J Bacteriol **184:**4510-4519.

258. **Willmitzer L, Sanchez-Serrano J, Buschfeld E, Schell J.** 1982. DNA from *Agrobacterium rhizogenes* in transferred to and expressed in axenic hairy root plant tissues. Mol Gen Genet **186:**16-22.

259. **Winans SC.** 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol Rev **56:**12-31.

260. **Young JPW, Crossman LC, Johnston AWB, Thomson NR, Ghazoui ZF, Hull KH, Wexler M, Curson ARJ, Todd JD, Poole PS, Mauchline TH, East AK, Quail MA, Churcher C, Arrowsmith C, Cherevach I, Chillingworth T, Clarke K, Cronin A, Davis P, Fraser A, Hance Z, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Sanders M, Simmonds M, Whitehead S, Parkhill J.** 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. Genome Biol **7:**R34.

261. **Zaenen I, Van Larebeke N, Teuchy H, Van Montagu M, Schell J.** 1974. Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J Mol Biol **86:**109-127.

262. **Zhang L, Kerr A.** 1991. A diffusible compound can enhance conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens*. J Bacteriol **173:**1867-1872.

263. **Zhang L, Kerr A.** 1993. Rapid purification of Ti plasmids from *Agrobacterium* by ethidium bromide treatment and phenol extraction. Lett Appl Microbiol **16:**265-268.

264. **Zhang L, Murphy PJ, Kerr A, Tate ME.** 1993. *Agrobacterium* conjugation and gene regulation by N-acyl-$_L$-homoserine lactones. Nature **362:**446-448.

265. **Zhang RG, Pappas T, Brace JL, Miller PC, Oulmassov T, Molyneaux JM, Anderson JC, Bashkin JK, Winans SC, Joachimiak A.** 2002. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. Nature **417:**971-974.

266. **Zhu J, Oger PM, Schrammeijer B, Hooykaas PJJ, Farrand SK, Winans SC.** 2000. The bases of crown gall tumorigenesis. J Bacteriol **182:**3885-3895.

267. **Zhu J, Winans SC.** 1998. Activity of the quorum-sensing regulator TraR of *Agrobacterium tumefaciens* is inhibited by a truncated, dominant defective TraR-like protein. Mol Microbiol **27:**289-297.

268. **Zhu J, Winans SC.** 1999. Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. Proc Natl Acad Sci USA **96:**4832-4837.

269. **Zhu J, Winans SC.** 2001. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. Proc Natl Acad Sci USA **98:**1507-1512.

270. **Zuckerkandl E, Pauling L.** 1965. Evolutionary divergence and convergence in proteins. *In* Bryson V, Vogel HJ (ed), Evolution of genes and proteins. Academic Press, New York.