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Agrobacterium plasmids encode structurally and functionally different loci for catabolism of agrocinopine-type opines

G. Thomas Hayman[1] * and Stephen K. Farrand[2]

[1] Department of Microbiology, Loyola University Medical Center, Maywood, Illinois 60153, USA
[2] Departments of Plant Pathology and Microbiology, University of Illinois, Urbana, IL 61801, USA

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Summary. *Agrobacterium tumefaciens* strains C58, T37, K827 and J73, *A. rhizogenes* strains A4 and 15834, and *A. radiobacter* strain K299 were all susceptible to agrocin 84 and this sensitivity was enhanced in each case by addition of agrocinopines A and B. Analysis of transconjugants showed that sensitivity of strain A4 to agrocin 84 was encoded by pArA4a and not by the rhizogenic plasmid, pRiA4. The *acc* region of the *A. tumefaciens* nopaline-type Ti plasmid pTiC58, contained on the recombinant plasmid pTHH206, hybridized strongly to restriction fragments of plasmids from strains T37, K827, J73 and K299. Hybridizing fragment patterns generated with *Bam*HI and *Eco*RI were identical among the four Ti plasmids while pAtK299 showed restriction fragment length polymorphisms at *acc* with the two enzymes. At moderate stringency, the pTiC58 *acc* region hybridized weakly to a single restriction fragment from the Ar plasmid of *A. rhizogenes* strain A4, but not to pTiBo542, which encodes catabolism of the closely related opines agrocinopines C and D. Plasmid pAtK84b of *A. radiobacter* strain K84 is induced for conjugal transfer by agrocinopines A and B. However, no hybridization was detected between this plasmid and *acc* from pTiC58 under conditions of moderate stringency. Like pTiC58, pAtK84b conferred transport of agrocinopines A and B on its host bacteria despite the absence of detectable sequence homology with the pTiC58-derived *acc* probe. However, unlike pTiC58, pAtK84b failed to confer sensitivity to or uptake of agrocin 84 on its bacterial host. These results indicate that at least four distinguishable systems exist for catabolism of the two agrocinopine opine families with the prototype locus, exemplified by *acc* from pTiC58, being strongly conserved among nopaline-type Ti plasmids.

* *Present address:* Microbial Properties Research, Northern Regional Research Center. USDA, Agricultural Research Service, 1815 North University Street, Peoria, IL 61604, USA

Offprint requests to: S.K. Farrand, Department of Plant Pathology, University of Illinois at Urbana/Champaign, N520 Turner Hall, 1102 South Goodwin Avenue, Urbana, IL 61801, USA

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Introduction

Crown gall tumors and hairy roots are plant neoplasias induced by pathogenic members of the genus *Agrobacterium*. They are characterized in part by production of novel, low molecular weight carbon compounds called opines (Tempé and Petit 1983), which generally are not found in normal plant tissues. The genes encoding their biosynthesis are acquired from the bacterium as part of the T-DNA which is transferred from the pathogen to the plant cell during infection (reviewed in Ream 1989). In the bacterium, the T-DNA forms part of a large virulence element called the Ti (Tumor inducing) or Ri (Root inducing) plasmid.

The opines may play a role in the natural biology of *Agrobacterium* by serving as unique and specific nutritional sources for the pathogenic bacteria. *Agrobacterium* strains can catabolize these compounds, and there is a strong correlation between the opine types produced by the neoplasias and those utilized by the inciting bacteria. In most cases the genes conferring catabolism of the opines are also encoded on the Ti or Ri plasmids but at loci separate from the T-regions. According to the Opine Concept, first proposed by Petit et al. (1978a), opines serve to foster the selective growth of the pathogen with the specificity being dictated by the catabolic genes encoded by the virulence plasmids.

Opines may play two additional roles in the biology of *Agrobacterium*. First, Ti plasmids are known to be conjugal elements (Kerr et al. 1977; Genetello et al. 1977; Beck von Bodman et al. 1989), but their transfer is strongly repressed. In those cases examined, certain classes of opines produced by the plant neoplasias act as specific inducers for Ti plasmid conjugal transfer (Petit et al. 1978b; Ellis et al. 1982b). Second, opines may

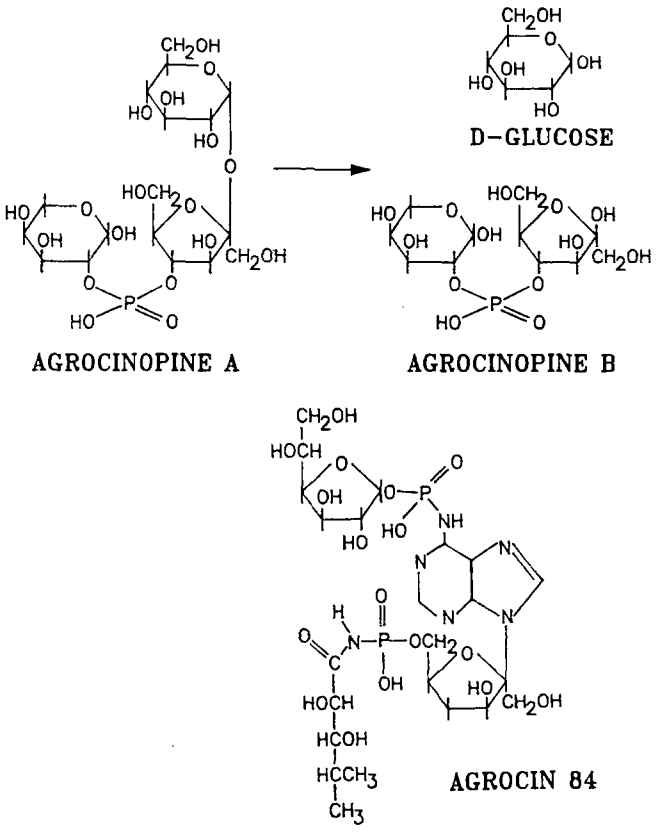

Fig. 1. Structures of agrocin 84 and agrocinopines A and B (from Murphy et al. 1981; Roberts et al. 1977; Ryder et al. 1984)

play a role in recognition between the *Agrobacterium* virulence functions and the host plant. Exogenous opines enhance 2- to 10-fold the acetosyringone-dependent induction of Ti plasmid-encoded virulence genes (Veluthambi et al. 1989).

A number of opine families have been identified with most being characterized as imine-linked conjugates. The exceptions are the two sugar phosphate opine classes, agrocinopines A/B and C/D. Both are phosphodiester conjugates; agrocinopine A containing arabinose and sucrose (Ryder et al. 1984) and agrocinopine C composed of glucose and sucrose (M.H. Ryder and M.E. Tate, personal communication). These opines, especially agrocinopines A and B (Fig. 1), are of interest for several reasons. First, nopaline-type Ti plasmids, such as pTiC58, which encode catabolism of the agrocinopine opines also confer sensitivity to a unique and highly specific antiagrobacterial antibiotic called agrocin 84 (Fig. 1; Farrand 1989). In fact, the agrocinopines were discovered during a directed search for the natural substrate of the Ti plasmid-encoded uptake system responsible for transporting agrocin 84 (Ellis and Murphy 1981). Physiological and genetic analyses showed that the antibiotic and the sugar phosphate opines are taken up by the same transport system (Ellis and Murphy 1981). This is significant because disease caused by *Agrobacterium* strains sensitive to agrocin 84 can be controlled in the field by inoculating plants with *A. radiobacter* strain K84, which produces agrocin 84 (Farrand 1989). Second, agrocinopines A and B are the conjugal opines for Ti plasmids such as pTiC58, pTiT37 and pTiK27 (Ellis et al. 1982b). When strain C58 donors are grown with agrocinopines A and B, Ti plasmid conjugal transfer increases from undetectable levels to frequencies approaching 10^{-1} transconjugants per input donor (Ellis et al. 1982b; Beck von Bodman et al. in preparation).

The *acc* locus encoding catabolism of agrocinopines A and B has been cloned and characterized from the prototype nopaline/agrocinopine Ti plasmid pTiC58 (Hayman and Farrand 1988). Mutations within this locus affecting uptake of the opines invariably abolish sensitivity to and transport of the antibiotic, confirming that this determinant encodes both phenotypes (Hayman and Farrand 1988). The locus has been mapped to a region of pTiC58 between 6 and 8 kb in size, and transcription is inducible by addition of agrocinopines A + B. The genes are expressed at a relatively high basal level, which explains why strains containing nopaline/agrocinopine-type Ti plasmids are sensitive to agrocin 84 in the absence of the inducer opines.

Susceptibility to agrocin 84 is not confined to agrobacteria harboring nopaline-type Ti plasmids. *A. rhizogenes* strains such as A4 and 15834 are also sensitive to agrocin 84 (Moore et al. 1979; White and Nester 1980), and hairy roots induced by these strains contain agrocinopine A (Petit et al. 1983). In addition, *A. tumefaciens* strain Bo542 incites tumors that produce a second family of sugar phosphate opines called agrocinopines C and D (Ellis and Murphy 1981). Strains harboring pTiBo542 exhibit sensitivity to agrocin 84, but only after induction by addition of agrocinopine C (Ellis and Murphy 1981). Finally, several strains of *A. radiobacter* also are sensitive to agrocin 84 (Roberts and Kerr 1974; our observations), implying that they are able to utilize one or more agrocinopine opines. As a variation on this theme, strain K84, the *A. radiobacter* biocontrol strain that produces agrocin 84, contains a large plasmid that encodes catabolism of nopaline. This element shows extensive DNA homology with pTiC58, although it does not encode T-DNA or *vir* functions (Clare et al. 1988). Like pTiC58, conjugal transfer of this opine catabolic plasmid is induced by agrocinopines A and B, implying that a determinant is also encoded for utilization of this opine family (Ellis et al. 1982b).

These observations suggest that genes encoding catabolism of the agrocinopine-type opines and sensitivity to agrocin 84 from various *Agrobacterium* plasmids may be different. In this study we used the cloned *acc* region from pTiC58 to determine the relationships between the various plasmid-associated agrocinopine catabolic loci. The results of DNA hybridization and physiological characterizations indicate that there are as many as four distinct plasmid-encoded determinants associated with agrocin 84 sensitivity or agrocinopine utilization.

Materials and methods

Bacterial strains and growth conditions. The strains and plasmids used in this work together with their sources are shown in Table 1. *Agrobacterium* strains were grown

Table 1. Bacterial strains and plasmids

Strain	Relevant plasmid(s)	Relevant genotype or phenotype[a]	Reference or source
Agrobacterium tumefaciens			
NT1	–	Agr84^r, Acp$^-$	Watson et al. (1975)
C58	pTiC58	Agr84^s, Acp$^+$, Tra$^+$	Zimmerer et al. (1966)
K439	pWI1000	Agr84^{ss}, Acp^c, Tra^c	Ellis et al. (1982a)
A281	pTiBo542	Agr84^s	Sciaky et al. (1978)
K827	pTiK27	Agr84^s	A. Kerr
T37	pTiT37	Agr84^s	Our collection
J73	pTiJ73	Agr73$^+$, Agr84^s	Webster et al. (1986)
C58C1RS (pArA4a)	pArA4a	Agr84^s, Rm, Sm, Mop, Moa, Aga	J. Tempé
C58C1RS (pRiA4)	pRiA4	Rm, Sm, Agr	J. Tempé
NT1 (pAtK84b)	pAtK84b	Agr84^r, Acp$^+$, Noc	This study
NT1 (pAgK84-A1)	pAgK84-A1	Agr84$^+$, Km	Farrand et al. (1985)
NT1 (pTiJ73)	pTiJ73	Agr84^s	Webster et al. (1986)
A. rhizogenes			
A4	pRiA4, pArA4a	Agr84^s, Mop, Moa, Agr, Aga	F. White
15834	pAr15834	Agr84^s, Acs, Agr	F. White
A. radiobacter			
K299	pAtK299	Agr84^s	A. Kerr
Plasmids			
pTiC58Tra^c		Agr84^{ss}, Acp$^+$, Tra^c	C. Kado
pTHH206		Agr84^s, Acp$^+$, Cm, Km	Hayman and Farrand (1988)

[a] Abbreviations: Acp, agrocinopine transport; Aga, agropinic acid catabolism; Agr, agropine catabolism; Agr73$^+$, agrocin 73 production; Agr84$^+$, agrocin 84 production; Agr84^r, agrocin 84 resistance; Agr84^s, agrocin 84 sensitivity; Agr84^{ss}, agrocin 84 supersensitivity; Moa, mannopinic acid catabolism; Mop, mannopine catabolism; Noc, nopaline catabolism; Tra^c, constitutive conjugal transfer; Tra$^+$, inducible conjugal transfer; Cm, chloramphenicol resistance; Km, kanamycin resistance; Rm, rifampicin resistance; Sm, streptomycin resistance

at 28° C. Media used were L broth (LB, Gibco), Nutrient Agar (NA, Difco), Stonier's medium (Stonier 1960), and AB minimal medium (Chilton et al. 1974). Tryptone yeast extract medium (TYE; Wanner 1986), yeast mannitol agar (YMA; Somasegaran and Hoben 1985), and yeast extract broth (YEB; Petit et al. 1978b) were used to grow *A. rhizogenes* strains. Beef extract, peptone, tryptone, and yeast extract used in the *A. rhizogenes* media were from Difco.

Chemicals. Partially purified preparations of agrocinopines A and B, agrocinopines C and D, and agrocin 84 were the gifts of M.H. Ryder.

Plasmid isolation. Plasmids under 50 kb in size were isolated from large (250 ml) cultures using the alkaline lysis method described by Maniatis et al. (1982). Purification of large (ca. 200 kb) plasmids was performed using the method of Casse et al. (1979) or, more consistently, by a method combining elements of the Maniatis and Casse protocols as follows: cells grown to late exponential phase in LB (200 ml) were harvested by centrifugation at $10000 \times g$ for 15 min at 4° C. The cell pellet was resuspended in 15 ml washing buffer (50 mM TRIS-HCl, 20 mM EDTA, 0.5 M NaCl, 0.05% sodium sarkosyl, pH 8.0) and centrifuged again. The cell pellet was gently resuspended in 15 ml ice-cold Solution 1 (25 mM TRIS-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0) and incubated on ice for 5 min. Freshly made Solution 2 (30 ml 0.2 N NaOH, 1% SDS) was added and the cell suspension mixed gently, followed by a 10 min incubation at room temperature (RT). Lysozyme (2 mg/ml) was added to Solution 1 in subsequent purifications if lysis of any given strain was incomplete. TRIS-HCl (2 M, pH 7.0, 7.5 ml) was added and the lysates were thoroughly and gently mixed. Sodium chloride (5 M, 7.5 ml) was added, the lysates were mixed gently, and left standing for 10 to 20 min at RT. Phenol saturated with 3% NaCl (60 ml) was added and the aqueous phase was extracted for 2–5 min by gentle agitation. The emulsions were centrifuged at $10000 \times g$ for 10 min at 4° C, and the aqueous phases transferred to clean centrifuge bottles. DNA was precipitated by addition of ice-cold ethanol (95%, two volumes) followed by storage at −20° C for 2 h to overnight. The precipitated DNA was collected by centrifugation at $10000 \times g$ for 20 min at 4° C. The pellets were washed once with ice-cold 70% ethanol (50 ml), and the bottles were recentrifuged. The ethanol was decanted, the pellets were air dried for about 5 min, and the DNA was redissolved in TES (30 mM TRIS-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) prior to centrifugation to equilibrium in two successive cesium chloride-ethidium bromide gradients as described previously (Farrand et al. 1981).

Restriction endonuclease digestion and agarose gel electrophoresis. Restriction endonuclease digestion of plasmid DNA was performed as described by Slota and Farrand (1982). Agarose gel electrophoresis conditions have been described elsewhere (Meyers et al. 1976; Slota and Farrand 1982).

High voltage paper electrophoresis. Opines were separated by high voltage paper electrophoresis on Whatman No. 1 or 3MM papers and visualized by alkaline silver

nitrate staining as described previously (Hayman and Farrand 1988).

Agrocin 84 and agrocinopine uptake. Uptake of agrocin 84 was detected by measuring disappearance of the toxic agent from culture supernatants following incubation with a high density of cells as described by Hayman and Farrand (1988). Agrocinopine transport was also determined by measuring disappearance of the opine from liquid cultures as outlined previously (Hayman and Farrand 1988).

Southern hybridizations. Equivalent amounts of plasmid DNA were digested with appropriate restriction enzymes and fragments separated by electrophoresis on 0.7% agarose gels. Probe DNA was radiolabeled by nick translation according to the instructions of the manufacturer [nick translation kit from BRL, ^{32}P-dCTP (650 Ci/mmol) from ICN]. DNA fragments separated by electrophoresis in agarose gels were transferred to membranes as described by Southern (1975) or according to the instructions of the manufacturer of the membrane used [GeneScreen™ Instruction Manual, New England Nuclear™ (Du Pont)]. Filters were hybridized with probe DNA under conditions of moderate stringency (ca. 20° C below T_m), as follows. The filters were prehybridized for 6 h at 42° C in a solution containing 50% deionized formamide, 10× Denhardt's solution [0.2% polyvinyl pyrrolidone (PVP, mol. wt. 40 000), 0.2% bovine serum albumin (BSA), 0.2% Ficoll (mol. wt. 400000); Denhardt 1966], 50 mM TRIS-HCl (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (mol. wt. 500000), 100 µg/ml sheared denatured salmon sperm DNA. Hybridizations were incubated overnight at 42° C in the same buffer containing probe DNA. The filters were washed twice at RT for 5 min each in a solution containing 0.3 M NaCl, 60 mM TRIS-HCl (pH 8.0), 2 mM EDTA, followed by two 30 min washes at 60° C with the same solution containing 1.0% SDS. Finally, the filters were washed twice at RT for 30 min each in a solution containing 0.03M NaCl, 6 mM TRIS-HCl (pH 8.0), 0.2 mM EDTA. Hybridization reactions were visualized by autoradiography.

Results

Agrocin 84 sensitivity and agrocinopine induction

Agrobacterium isolates that are sensitive to agrocin 84 or induce plant neoplasias containing agrocinopines include *A. tumefaciens* strains C58, T37, J73, K827, and A281, *A. radiobacter* strain K299, and *A. rhizogenes* strains A4 and 15834 (Ellis and Murphy 1981; Moore et al. 1979; Murphy and Roberts 1979; Petit et al. 1983; Roberts and Kerr 1974; Webster et al. 1986; White and Nester 1980). These strains were tested for sensitivity to agrocin 84 and for the effect of agrocinopines on that reaction. Strains NT1 or C58C1RS containing plasmids from strains J73, K84 and A4 were also analyzed.

Table 2. Opine-inducible agrocin 84 sensitivity of *Agrobacterium* strains containing various Ti, At, Ri, and Ar plasmids[a]

Strain	Agrocin 84 reaction[b]	Induction by[c]	
		Acp A/B	Acp C/D
C58	S	+	−
T37	S	+	−
J73	S	+	−
NT1(pTiJ73)	S	+	−
K827	S	+	−
K299	S	+	−
NT1(pAtK84b)	R	−	−
A281	S*	−	+
C58C1RS(pArA4a)	S	+	−
C58C1RS(pRiA4)	R	−	−
A4	S	+	N.D.
15834	S	+	N.D.

[a] All phenotypes were determined by the plate bioassay method as described in the Materials and methods

[b] S, agrocin 84-sensitive; R, agrocin 84-resistant; S*, agrocin 84-sensitive only in the presence of inducer opines

[c] Acp, agrocinopine; +, sensitivity to agrocin 84 induced by opine; −, sensitivity to agrocin 84 not induced by opine; N.D., not determined

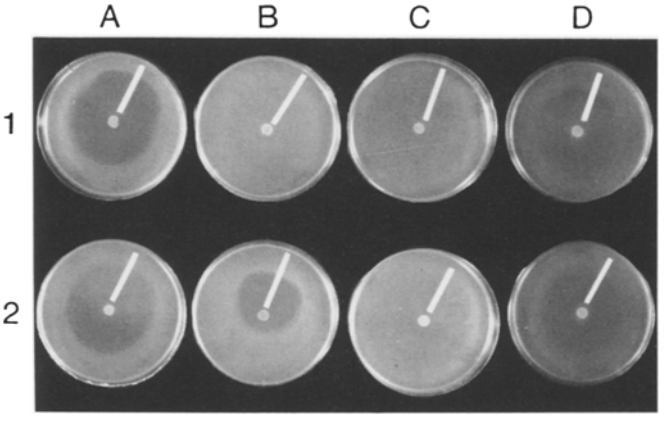

Fig. 2. Agrocin 84 bioassays of selected *Agrobacterium* strains. Assays were performed as described by Hayman and Farrand (1988). Stonier's medium plates were spotted with agrocin 84 producer strain NT1(pAgK84-A1) and overlaid with indicator strains as follows: A, C58; B, A281; C, NT1(pAtK84b); D, C58C1RS(pArA4a). Paper strips were impregnated with: row 1, agrocinopines A + B; row 2, agrocinopines C + D

Virulent nopaline-type strains C58, T37 and K827 were susceptible to agrocin 84, and in each case sensitivity was accentuated by addition of agrocinopines A+B (Table 2, see Fig. 2A). The tumorigenic South African nopaline-type strain, J73, and a strain NT1 transconjugant harboring pTiJ73 displayed this same reaction to the antibiotic (Table 2). The nontumorigenic nopaline-catabolizing *A. radiobacter* strain, K299, also exhibited susceptibility to agrocin 84 which was accentuated by agrocinopines A+B (Table 2). Transconjugant strain A281, which harbors the agropine-type Ti plasmid, pTiBo542, and NT1 containing the nopaline catabolic plasmid pAtK84b of *A. radiobacter* strain K84 were resistant

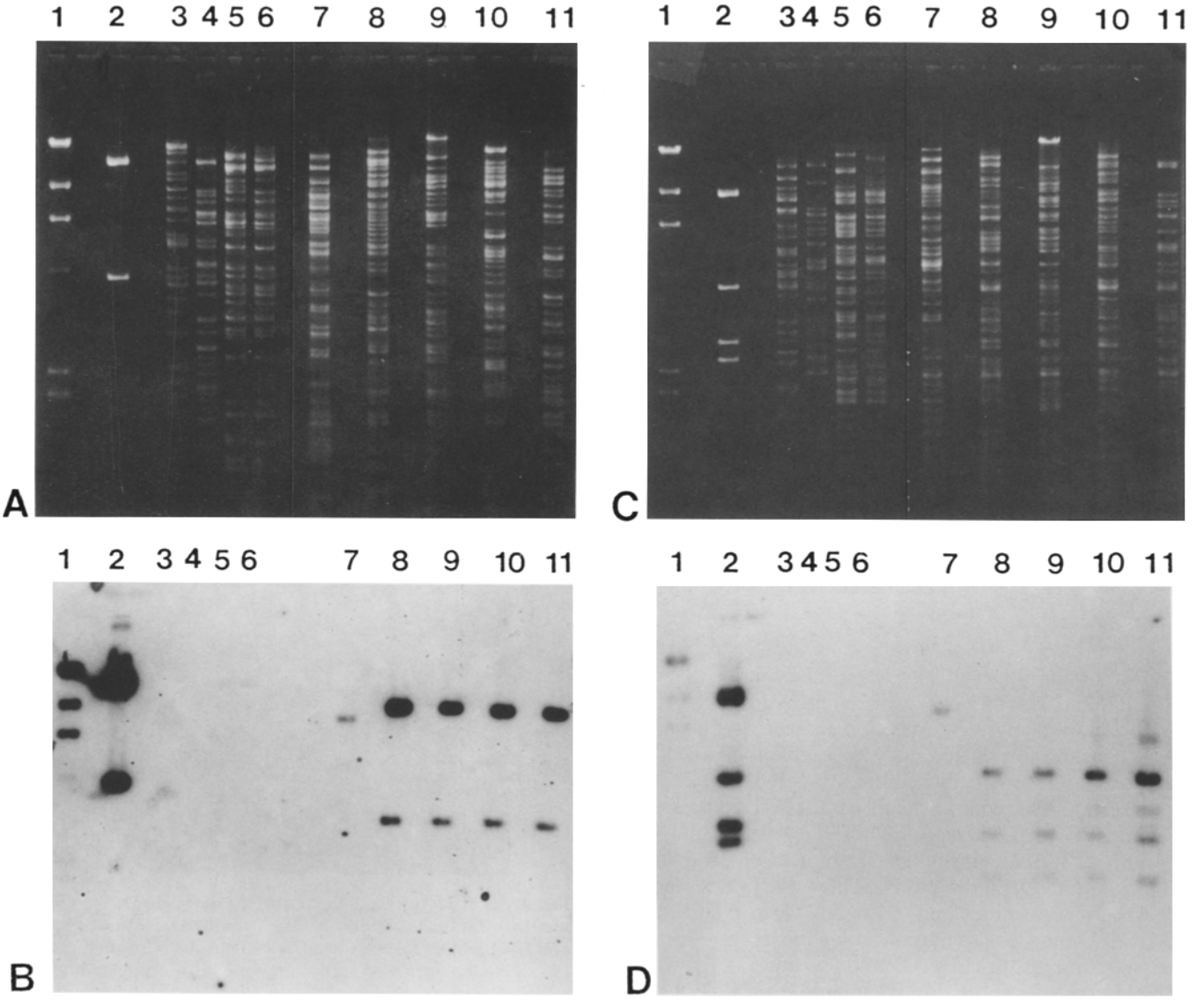

Fig. 3A–D. Electrophoretic and Southern analysis comparing the *acc* region of pTiC58 with regions on various *Agrobacterium* plasmids. Approximately equal amounts of plasmid DNAs were digested with *Bam*HI (**A**, **B**) or *Eco*RI (**C**, **D**), subjected to agarose gel electrophoresis, and stained with ethidium bromide (**A**, **C**) as described in the Materials and methods. The DNA fragments were transferred to GeneScreen™ filters and probed with a mixture of pTHH206 and *Hin*dIII-cleaved lambda DNA labeled with ^{32}P (**B**, **D**). Lanes contain: 1, λ *Hin*dIII; 2, pTHH206; 3, pTiBo542; 4, pAtK84b; 5, 15834 total plasmid DNA; 6, pArA4a; 7, K299 plasmid DNA; 8, pTiK27; 9, J73 plasmid DNA; 10, pTiT37; 11, pTiC58

to the antibiotic (Table 2, Fig. 2B and C). Agrocin 84 sensitivity was not induced by agrocinopines A+B in either strain. However, susceptibility of A281 to the antibiotic was induced by agrocinopines C+D (Table 2, Fig. 2B). This opine family produced no effect on the agrocin-resistant phenotype of NT1(pAtK84b) (Fig. 2C) and also did not accentuate agrocin 84 sensitivity shown by strains that responded to agrocinopines A+B (Table 2).

Agrobacterium rhizogenes strains 15834 and A4 both displayed sensitivity to agrocin 84 which was further inducible by agrocinopines A+B (Table 2). Analysis of strain NT1 transconjugants containing either the Ri or the Ar plasmid from strain A4 demonstrated that susceptibility is encoded by pArA4a, rather than by the virulence plasmid (Fig. 2D, Table 2). The zone of growth inhibition exhibited by NT1(pArA4a) was substantially more turbid and less distinct compared to that of strain C58. However, like the latter, sensitivity conferred by the *A. rhizogenes* plasmid was enhanced by agrocinopines A+B (compare Fig. 2 panels A and D). Agrocinopines C+D had no effect on the agrocin sensitivity phenotypes of strains A4, 15834 or NT1(pArA4a) (Table 2).

Hybridization analysis of acc *loci*

Southern hybridization analyses with pTHH206 as the probe were used to assess relatedness between the *acc* region of pTiC58 and plasmids purified from other

Table 3. Classes of *acc* regions

Class	Source	Characteristics		
		Agrocinopine transport[a]	Agrocin 84 sensitivity[b]	Sequence similarity to pTiC58[c]
I	pTiC58	+	S	Prototype
	pTiT37	+	S	+
	pTiK27	+	S	+
	pTiJ73	N.D.	S	+
	pAtK299	N.D.	S	+[d]
II	pArA4a	N.D.	S	±
III	pTiBo542	−	S*	−
IV	pAtK84b	+	R	−

[a] +, plasmid confers transport of agrocinopines A and B; −, agrocinopine A and B transport not conferred; N.D., not determined

[b] S, agrocin 84-sensitive; S*, agrocin 84-sensitive only in the presence of opine; R, agrocin 84-resistant

[c] +, strong hybridization to cloned *acc* probe; ±, weak hybridization to probe; −, no detectable hybridization to probe

[d] Homology displayed restriction fragment length polymorphisms

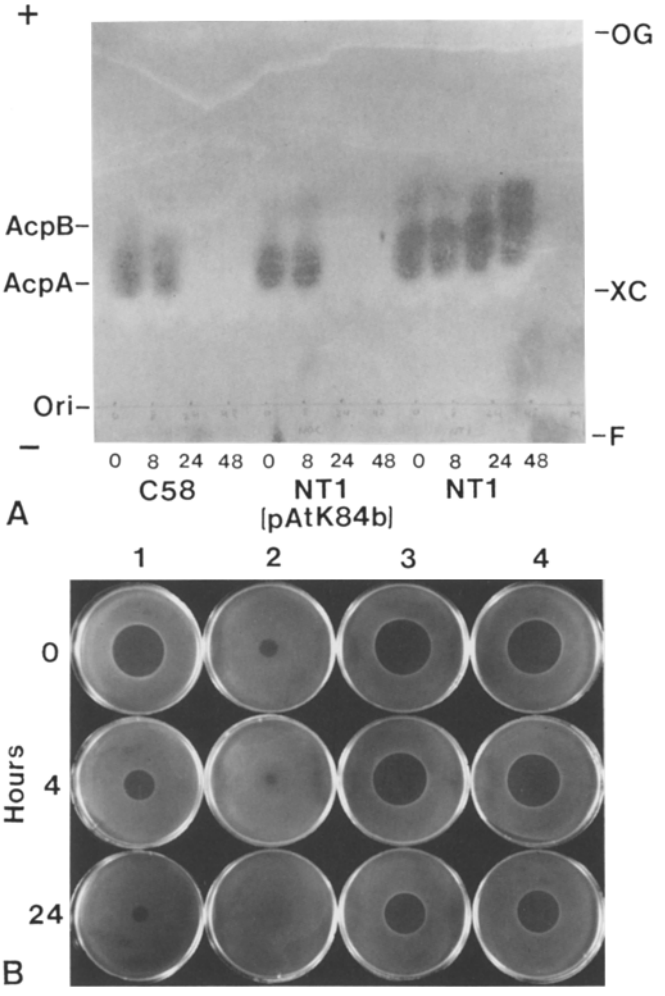

Fig. 4A and B. Agrocinopine (Panel A) and agrocin 84 (Panel B) uptake by strains C58 and NT1(pAtK84b). **A** Strains C58, NT1, and NT1(pAtK84b) were inoculated into ATN medium cultures (30 µl) containing 3 mM agrocinopines A + B. Samples (5 µl) were removed at the timepoints indicated (in hours) at the bottom of the figure and subjected to high voltage paper electrophoresis (HVPE) in formic acid/acetic acid buffer at pH 1.8. Agrocinopines were visualized by alkaline silver nitrate staining. HVPE conditions were as described previously (Hayman and Farrand 1988). **B** Strains C58, NT1(pTiC58Tra^c), NT1, and NT1(pAtK84b) were tested for agrocin 84 transport. Following growth to mid-exponential phase in Stonier's medium, cells were concentrated 50-fold and 27 µl incubated with 3 µl partially purified agrocin 84 as previously described (Hayman and Farrand 1988). Samples (10 µl) were removed at the time intervals shown at the left, centrifuged and the supernatants spotted on Stonier's plates. Following absorption of the samples into the agar, the plates were sterilized by exposure to chloroform vapors, and covered with 3 ml of top agar (0.7% agar in 20 mM potassium phosphate buffer, pH 7.0) seeded with *A. tumefaciens* strain NT1(pTiC58Tra^c) as the indicator strain. Supernatants spotted in the center of the plates are from cultures of strains: 1, C58; 2, NT1(pTiC58Tra^c); 3, NT1; 4, NT1(pAtK84b)

Agrobacterium strains. The nopaline-type Ti plasmids pTiK27 and pTiT37, as well as plasmid DNA from strain J73 all displayed restriction enzyme digestion patterns considerably different from each other and from that of pTiC58 (Fig. 3A and C, lanes 8–11). Despite these differences, when probed with pTHH206 all three plasmids yielded strongly hybridizing fragments with mobilities indistinguishable from those of pTiC58 (Fig. 3B and D, lanes 8–11). The only other plasmid showing strong homology with *acc* from pTiC58 was pAtK299, the opine catabolic plasmid from *A. radiobacter* strain K299 (Fig. 3B and D, lane 7). However, the patterns of hybridizing fragments from this plasmid differed from those of pTiC58 and the other nopaline-type Ti plasmids. Likewise, the restriction endonuclease banding pattern of strain K299 plasmid DNA in ethidium bromide-stained agarose gels differed from the banding patterns of plasmid DNA from strains T37, K827, J73 and C58 (Fig. 3A and C).

No hybridization was detected between pTHH206 and pTiBo542 (Fig. 3B and D, lane 3) or pAtK84b (Fig. 3B and D, lane 4). Single very faintly hybridizing fragments with indistinguishable mobilities were observed in lanes containing total plasmid DNA from *A. rhizogenes* strain 15834 or pArA4a digested with *Bam*HI (Fig. 3B, lanes 5 and 6).

Comparison of pTiC58 and pAtK84b acc *regions*

Agrocinopines A+B induce the conjugal transfer of pAtK84b, suggesting that this plasmid encodes utilization of the sugar phosphate opines (Ellis et al. 1982b). However, there is no significant homology between pTiC58 *acc* and pAtK84b (Fig. 3B and D, lane 4 and data not shown). Furthermore, strains containing this plasmid are resistant to agrocin 84, even in the presence of the opine, indicating that the agrocinopine catabolic determinant does not confer susceptibility to this antibiotic. To examine the specificity of the opine transport system encoded by this plasmid, we determined whether pAtK84b confers uptake of agrocinopines A+B or of

agrocin 84. Strains NT1, C58 and NT1(pAtK84b) were incubated over a 48 h period at 28° C in medium containing 3 mM agrocinopines A+B as sole carbon source. High voltage paper electrophoretic analysis of culture supernatants sampled at various times showed that strains C58 and NT1(pAtK84b) removed the opines from the culture medium (Fig. 4A). Strain NT1, as expected, failed to utilize the opines (Fig. 4A). These strains were also tested for their ability to remove agrocin 84 from the culture medium. As shown in Fig. 4B, the antibiotic disappeared from culture supernatants inoculated with strains C58 and a spontaneous C58 mutant derepressed for *acc* (Ellis et al. 1982a). As we have shown previously (Hayman and Farrand 1988), the mutant accumulates agrocin 84 at a rate considerably faster than that shown by wild-type strain C58. However, like the Ti plasmid-less strain NT1, the transconjugant containing pAtK84b failed to remove agrocin 84 from the culture medium over the time period tested.

Discussion

The physiological and hybridization studies reported here indicate that determinants from *Agrobacterium* plasmids encoding transport or utilization of the agrocinopine opines can be grouped into at least four classes (Table 3). The first, exemplified by the prototype *acc* locus of pTiC58, encodes transport and utilization of agrocinopines A and B and also sensitivity to agrocin 84 (Ellis and Murphy 1981; Ellis et al. 1982b; Hayman and Farrand 1988; Hayman and Farrand, in preparation). This locus is expressed at a high basal level and is further inducible by addition of the A and B-type sugar phosphate opines (Hayman and Farrand 1988). The pTiC58-type *acc* locus is functionally and structurally conserved in other nopaline-type Ti plasmids including pTiT37, pTiK27, and pTiJ73. Restriction fragments from these plasmids that hybridize to pTHH206 show mobilities indistinguishable from those of the prototype *acc* region (Fig. 3). The class I locus is also closely related to a region on the opine catabolic plasmid of *A. radiobacter* strain K299. This strain exhibits sensitivity to agrocin 84 which is further inducible by agrocinopines A and B (Table 2); however, differences in the sizes of the hybridizing fragments show that the *acc* locus encoded by pAtK299 has diverged to some degree from that of pTiC58 (Fig. 3).

The second class, represented by the *A. rhizogenes* plasmid pArA4a, also encodes sensitivity to agrocin 84 which can be further accentuated by exposure to agrocinopines A+B (Fig. 2D, Table 2). The presence of an *acc* determinant in these *A. rhizogenes* isolates is consistent with the observation that hairy roots induced by strain A4 contain agrocinopine A (Petit et al. 1983). However, only a single fragment from pArA4a shows detectable homology with *acc* from pTiC58 and the hybridization is very weak (Fig. 3B). Furthermore, as indicated by the plate bioassay method, the phenotype for agrocin 84 sensitivity conferred by the *A. rhizogenes* plasmid differs from that associated with the nopaline-type plasmids (compare Fig. 2 plates A and D).

The third class of *acc* determinant is represented by pTiBo542. This Ti plasmid encodes sensitivity to agrocin 84, but only following addition of agrocinopines C and D (Fig. 2B). Agrocinopines A and B have no effect on the agrocin 84 sensitivity phenotype conferred by pTiBo542. Agrocinopines C and D are structurally similar, but not identical to agrocinopines A and B (Ellis and Murphy 1981; M.H. Ryder and M.E. Tate, personal communication). Given this difference in substrates, it is possible that the *acc* locus of pTiBo542 arose independently from the agrocinopine A and B catabolic genes. This interpretation is strengthened by the observations that (i) strains harboring pTiBo542 do not take up agrocinopines A and B (Table 3) and (ii) no hybridization was detected between this Ti plasmid and the pTiC58 *acc* probe at the stringency tested. It is interesting to note that while the pTiBo542 *acc* transport system does not recognize agrocinopines A and B, it apparently does transport agrocin 84. This might indicate that the agrocinopine A/B and C/D transport systems recognize different portions of the antibiotic molecule. Alternatively, some structural feature of agrocin 84 held in common with the two opine classes might be recognized by each of the two, otherwise opine-specific, transport systems. In this case transport specificity would reside in other portions of the opine molecules.

The fourth *acc* class is exemplified by pAtK84b, the opine catabolic plasmid present in *A. radiobacter* strain K84. The *acc* determinant on this plasmid encodes uptake of agrocinopines A and B (Fig. 4A), and these opines also induce conjugal plasmid transfer (Ellis et al. 1982b). In these respects the opine-associated phenotypes of strains K84 and C58 are similar (Fig. 4A; Ellis et al. 1982b; Hayman and Farrand 1988). However, pAtK84b shows no detectable hybridization with the pTiC58 *acc* probe (Fig. 3 and data not shown), does not confer sensitivity to agrocin 84 in either the presence or absence of the agrocinopine opines (Fig. 2C), and does not specify uptake of the antibiotic (Fig. 4B). Clearly, the *acc* locus of pAtK84b is related only distantly, if at all, to pTiC58 *acc*.

The weak homology between the pTiC58 *acc* probe and pArA4a is difficult to interpret. The probe used in these studies contains approximately 3 kb of Ti plasmid DNA sequences that flank *acc* and from our data it is not possible to determine which regions of pTHH206 are involved in the weak hybridization with this *A. rhizogenes* plasmid. Given the differences in phenotypes and the absence of significant homologies with pTHH206, it is possible that the class II, III, and IV catabolic systems each arose independently or diverged early on from the prototype pTiC58 *acc* determinant. This interpretation is strengthened by the contrasting high degree of conservation at *acc* exhibited by the class I plasmids.

Conservation at *acc* among the class I Ti and At plasmids in turn contrasts with the lack of similarity between these plasmids at the level of restriction fragment pattern (Fig. 3A and C). While *Agrobacterium* virulence plasmids often share large regions of sequence homology, especially at *vir* and T-DNA loci (Engler

et al. 1981; Huffman et al. 1984; Komari et al. 1986; Hood et al. 1986), it is clear from the dissimilarities in restriction profiles (Fig. 3; also Sciaky et al. 1978) that the nopaline-type Ti plasmids are considerably divergent. This is in contrast to the Ti plasmids from independent octopine-type isolates such as strains A6, Ach5, B6, 15955, and R10; these elements are almost identical at the restriction fragment pattern level (Sciaky et al. 1978; our unpublished observations). Among the nopaline-type Ti plasmids, the *acc* locus is conserved across large geographical distances; strains C58 and T37 are both United States isolates (Sciaky et al. 1978), while strain J73 comes from South Africa (Webster et al. 1986) and strain K27, the source of the Ti plasmid in strain K827, was isolated in South Australia (Kerr 1972). This suggests that phenotypes conferred by the *acc* locus are important to *Agrobacterium* strains harboring these plasmids.

Unlike *A. tumefaciens* systems, opine catabolic functions in some *A. rhizogenes* strains are not necessarily contained on the virulence plasmids. For example, with strains such as A4 and 15834, genes encoding catabolism of mannopinic acid, mannopine, and agropinic acid are encoded by the ancillary Ar plasmids (Petit et al. 1983). Our results indicate that determinants for agrocinopine A and B catabolism also are associated with the Ar plasmid in strain A4. This emphasizes the importance of these so-called opine catabolic plasmids. The fact that *acc* determinants are also associated with large, non-oncogenic plasmids in *A. radiobacter* strains K299 and K84 suggests that the ability to catabolize opines plays a major role in the natural biology of non-oncogenic *Agrobacterium* species.

Our results demonstrate that the opine catabolic plasmid of strain K84 encodes an *acc* determinant which differs substantially from the pTiC58 locus in its genetic composition and the phenotypic properties it confers. This is interesting considering that pAtK84b otherwise shows considerable overall homology with pTiC58 as detected by renaturation kinetic analysis (Merlo and Nester 1977) and Southern hybridizations (Clare et al. 1988). However, despite their differences, these loci serve the same purpose; namely to allow uptake of the agrocinopine opines, presumably for nutritional purposes, and also to induce conjugal transfer of the plasmid (Ellis et al. 1982b). That the pAtK84b-specific *acc* determinant fails to confer sensitivity to agrocin 84 may be due to the fact that it is present in precisely the *Agrobacterium* strain that produces this antibiotic. While the agrocinogenic plasmid, pAgK84, also encodes agrocin 84 immunity functions (Farrand et al. 1985), the inactivity of the pAtK84b *acc* locus with respect to agrocin 84 may represent another level of protection against the action of this antibiotic.

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References

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

Casse F, Boucher C, Julliot JS, Michel M, Denarié J (1979) Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J Gen Microbiol 133:229–242

Chilton M-D, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

Clare BG, Kerr A, Jones DG (1988) Nopaline catabolism plasmid of the biological control *Agrobacterium* strains K84 and K1026. Abstr 5th Int Congr Plant Pathol, Abstr no 2–47, p 107

Denhardt DT (1966) A membrane-filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–650

Ellis JG, Murphy PJ (1981) Four new opines from crown gall tumors – their detection and properties. Mol Gen Genet 181:36–43

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

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

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

Farrand SK (1989) *Agrobacterium radiobacter* strain K84: A model biocontrol system. In: RR Baker, PE Dunn (eds) New directions in biological control. Liss, New York, pp 679–691

Farrand SK, Kado CI, Ireland CR (1981) Suppression of tumorigenicity by the IncW R-plasmid pSa in *Agrobacterium tumefaciens*. Mol Gen Genet 181:44–51

Farrand SK, Slota JE, Shim J-S, Kerr A (1985) Tn*5* 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

Genetello C, van Larebeke N, Holsters M, Depicker A, Van Montagu M, Schell J (1977) Ti plasmids of *Agrobacterium* as conjugative plasmids. Nature 265:561–563

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

Hood EE, Chilton WS, Chilton M-D, Fraley RT (1986) T-DNA and opine synthetic loci in tumors incited by *Agrobacterium tumefaciens* A281 on soybean and alfalfa plants. J Bacteriol 168:1283–1290

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

Kerr A (1972) Biological control of crown gall: seed inoculation. J Appl Bacteriol 35:493–497

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

Komari T, Halperin W, Nester EW (1986) Physical and functional map of supervirulent *Agrobacterium tumefaciens* tumor-inducing plasmid pTiBo542. J Bacteriol 166:88–94

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Merlo D, Nester EW (1977) Plasmids in avirulent strains of *Agrobacterium*. J Bacteriol 129:76–80

Meyers JA, Sanchez D, Elwell LP, Falkow S (1976) Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 127:1529–1537 [Erratum (1977) J Bacteriol 129:1171]

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

Murphy PJ, Roberts WP (1979) A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. J Gen Microbiol 114:207–213

Murphy PJ, Tate ME, Kerr A (1981) Substituents at N^6 and C-5′ control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in bacteria. Eur J Biochem 115:539–543

Petit A, Dessaux Y, Tempé J (1978a) The biological significance of opines: I. A study of opine catabolism by *Agrobacterium tumefaciens*. In: Ridé M (ed) Proc 4th Int Conf Plant Pathog Bacteriol, INRA, Angers, pp 143–152

Petit A, Tempé J, Holsters M, van Montagu M, Schell J (1978b) Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature 271:570–571

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

Ream W (1989) *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annu Rev Phytopathol 27:583–618

Roberts WP, Kerr A (1974) Crown gall induction: serological reactions, isozyme patterns and sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of *Agrobacterium radiobacter*. Physiol Plant Pathol 4:81–92

Roberts WP, Tate ME, Kerr A (1977) Agrocin 84 is a 6-N-phosphoramidate of an adenine nucleotide analogue. Nature 265:379–380

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

Sciaky D, Montoya AL, Chilton M-D (1978) Fingerprints of *Agrobacterium* Ti-plasmids. Plasmid 1:238–253

Slota JE, Farrand SK (1982) Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. Plasmid 8:175–186

Somasegaran P, Hoben HJ (1985) Methods in legume-*Rhizobium* technology. NifTAL Project, Hawaii, p 273

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Stonier T (1960) *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. J Bacteriol 79:889–898

Tempé J, Petit A (1983) La piste des opines. In: Pühler A (ed) Molecular genetics of the bacteria-plant interaction. Springer-Verlag, Berlin, pp 14–32

Veluthambi K, Krishnan M, Gould JH, Smith RH, Gelvin SB (1989) Opines stimulate induction of the *vir* genes of the *Agrobacterium tumefaciens* Ti plasmid. J Bacteriol 171:3696–3703

Wanner BL (1986) Bacterial alkaline phosphatase clonal variation in some *Escherichia coli* K-12 *phoR* mutant strains. J Bacteriol 168:1366–1371

Watson B, Currier TC, Gordon MP, Chilton M-D, Nester EW (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. J Bacteriol 123:255–264

Webster J, Dos Santos M, Thompson JA (1986) Agrocin-producing *Agrobacterium* strain active against grapevine isolates. Appl Environ Microbiol 52:217–219

White FF, Nester EW (1980) Hairy root: Plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J Bacteriol 141:1134–1141

Zimmerer RP, Hamilton RH, Pootjes C (1966) Isolation and morphology of temperate *Agrobacterium tumefaciens* bacteriophage. J Bacteriol 92:746–750

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