Genetic Mapping and Characterization of acc, the Agrocinopine-Agrocin 84 Region, on pTiC58, the Nopaline-Type Ti Plasmid of Agrobacterium tumefaciens Strain C58

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OF ace, THE AGROGINOPINE-AGROCIN 84 REGION,
ON pTiC58, THE Nopaline-TYPE Ti Plasmid
OF Agrobacterium tumefaciens STRAIN C58

by

G. Thomas Hayman

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
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This work is dedicated to my family, whose love, understanding, and support have been a constant source of strength, to Cathryn Kaake and John Turner, who will sadly never see this, and to Peter Allerup. May I face life's problems with the same courage and determination you and your family have shown, my friend.
The author, George Thomas Hayman, is the son of Eugene J. Hayman and Norma (Kaake) Hayman. He was born on August 29, 1958 in New London, Connecticut.

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LIST OF ABBREVIATIONS

A: absorbance
AB: *Agrobacterium* minimal medium
   (contains nitrogen)
Acp: agrocinopine transport
AcpA: agrocinopine A
AcpB: agrocinopine B
Acs: agrocinopine biosynthesis
Aga: agropinic acid catabolism
Agr: agropine catabolism
Agr73*: agrocin 73 production
Agr73*: agrocin 73 sensitivity
Agr84*: agrocin 84 production
Agr*, Agr84*: agrocin 84 resistance
Agr*, Agr84*: agrocin 84 sensitivity
Agr84**: agrocin 84 supersensitivity
Ap: ampicillin resistance
Ape: bacteriophage API exclusion
AT: *Agrobacterium* minimal medium
   (lacks nitrogen)
ATN: *Agrobacterium* minimal medium
   (contains nitrogen)
ATP: adenosine triphosphate
B-Gal: B-galactosidase
BPB: bromphenol blue
BRL: Bethesda Research Laboratories
ca.: circa
Cb: carbenicillin resistance
CIP: calf intestinal alkaline phosphatase
cm: centimeter
Cm: chloramphenicol resistance
dCTP: deoxycytosine triphosphate
DMSO: Dimethyl sulfoxide
DNA: deoxyribonucleic acid
EB: extraction buffer
EC: *Escherichia coli* minimal medium
EDTA: ethylenediamine tetra-acetic acid
Em: erythromycin resistance
Fig.: figure
Gly*: glycine auxotrophy
Gm: gentamicin resistance
h: hour(s)
HVPE: high voltage paper electrophoresis
IncPl: incompatibility group Pl
IncQ: incompatibility group Q
IncW: incompatibility group W
IPTG: isopropyl-β-D-thio-galactopyranoside
kb: kilobase(s)
kD: kilodaltons
Km: kanamycin resistance
L: liter
LA: Luria agar
LB: Luria broth
LSB: Laemmli sample buffer
LTE: 10 mM Tris-HCl/1 mM EDTA, pH 8.0
µg: microgram
µl: microliter
M: molar
Met*: methionine auxotrophy
mg: milligram
MH: Mueller Hinton agar
min: minute(s)
ml: milliliter
mm: millimeter
mM: millimolar
Moa: mannopinic acid catabolism
Mop: mannopine catabolism
NA: nutrient agar
Nm: neomycin resistance
nmole: nanomoles
Noc: nopaline catabolism
Nx: nalidixic acid resistance
Occ: octopine catabolism
OD: optical density
OG: orange G
ONPG: ortho-nitrophenyl β-D-galactopyranoside
Ori: origin of electrophoresis
PCI: phenol/chloroform/isoamyl alcohol, 25:24:1
PPO: 2,5-diphenyloxazole
Pro*: proline auxotrophy
ref.: reference
RFLP: restriction fragment length polymorphism
Rm: rifampicin resistance
RT: room temperature
SDS: sodium dodecyl sulfate
Sm: streptomycin resistance
Sp: spectinomycin resistance
SSC: Standard Saline Citrate
TBE: Tris-HCl/borate/EDTA buffer, pH 8.0
Tc: tetracycline resistance
Tc*: tetracycline sensitive
TD: tracking dye
TE: 50 mM Tris-HCl/20 mM EDTA, pH 8.0
TEMED: N,N,N',N'-tetramethylethylenediamine
TES: 30 mM Tris-HCl/5 mM EDTA/50 mM NaCl, pH 8.0
Tra*: constitutive conjugal transfer
Tra': conjugal transfer negative
Tra', Tra*: inducible conjugal transfer
Tris: Tris(hydroxymethyl)aminomethane
TYE: tryptone yeast extract medium
XC: xylene cyanole
X-gal: 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
YEB: yeast extract beef extract medium
YMA: yeast mannitol agar
LIST OF GENETIC DESIGNATIONS

acc: agrocinopine catabolism
acs: agrocinopine biosynthesis
agr: agrocin 84 sensitivity
iaa: indole-3-acetic acid biosynthesis
ipt: isopentenyladenosine-5-monophosphate biosynthesis
lac: lactose catabolism
lrm: lambda receptor/maltoporin
leu: leucine biosynthesis
mob: mobilization
noc: nopaline catabolism
nos: nopaline biosynthesis
occ: octopine catabolism
ocs: octopine biosynthesis
onc: oncogenicity
ori: origin of conjugal transfer (T) or vegetative replication (V)
pol: DNA polymerase
pro: proline biosynthesis
psc: phosphorylated sugar catabolism
rec: recombination
ser: serine biosynthesis
tac: trp/lac promoter
thi: thiamine biosynthesis
tra: conjugal transfer
trp: tryptophan biosynthesis
vir: virulence
1.1. **Crown gall and the Opine Concept.**

*Agrobacterium tumefaciens* and *A. rhizogenes* are gram-negative soil bacteria that cause cancerous growths, called crown galls and hairy roots, respectively, on many dicotyledonous plants (144, 160). Crown gall is responsible for considerable crop losses worldwide (3, 100, 137). *A. tumefaciens* has been called the third most important bacterial plant pathogen in the United States after *Xanthomonas vesicatoria* (bacterial spot) and *Erwinia carotovora* (soft rot; ref. 96).

How this organism causes disease has been studied in depth over the past several decades. The early data that accumulated on the crown gall and hairy root disease processes centered around two areas, (i) the metabolism of crown gall tumors, and (ii) the genomes of agrobacteria. Discoveries in these areas led to a unifying proposal, the Opine Concept, which provided a satisfying teleology and guidelines for subsequent experimentation (140).

In the 1950’s, novel amino acids, later called opines, were discovered in crown gall tumors (116). Among the first described were octopine and nopaline (66, 121, 122, 123). Researchers found that opines have several unique properties. They are catabolized by virulent agrobacteria (121, 139), and aside from the presence of octopine in octopus muscle (131), they are found only in crown gall tumors, not in untransformed plants (67, 65). Since then other workers have found octopine and nopaline in wild-type plant tissues, but the opines are
present at low levels and their synthesis requires high levels of arginine (100 mM) in the growth medium (27).

Several correlations were observed between the opine classes found in tumors and the inciting bacterial strains. First, certain isolates always induced tumors containing octopine, the tumors of other agrobacteria always contained nopaline, and no opines could be found in tumors induced by the so-called null-type strains (139, 156). Also, most isolates causing tumors containing octopine could catabolize only octopine, and the majority of strains inducing galls containing nopaline could utilize only this opine. Most null strains could not utilize either octopine or nopaline (139). Catabolism of opines was also positively correlated with virulence.

Early work by Kerr (97, 98) demonstrated that the factor responsible for tumorigenesis could be transferred from strain to strain in planta. Nontumorigenic recipient isolates, marked by mutation to drug resistance, were inoculated onto plant tumors induced by inoculation of donor strains two weeks previously. After six weeks the tumors were macerated and spread on selective media that allows growth of only the recipient strain. Colonies were obtained that were virulent when tested for tumor production on plants, indicating that the recipient strain had received a virulence factor from the donor. At about the same time, Hamilton and Fall (69) discovered that a virulence factor could be lost. By incubating virulent strains at elevated temperatures, they could be converted to stable avirulent strains. They found that inocula taken from cultures incubated at 36°C for increasing periods of time contained increasing proportions of avirulent cells. While a high-frequency mutation might explain this,
the authors thought it more likely that the isolates had lost a virulence factor.

In looking for a candidate for this virulence factor, Zaenen et al. (202) were the first to discover large (ca. 200 kilobase pair) plasmids, called Ti plasmids, contained within virulent agrobacteria, and absent from avirulent isolates. These elements were identified by centrifuging cellular DNA through sucrose gradients or to equilibrium in cesium chloride-ethidium bromide gradients. Van Larebeke et al. (187) combined the approaches of Hamilton and Fall (69) and Zaenen et al. (202), showing that after extended incubation at 37°C, all the isolates of the virulent strain C58 tested had become avirulent. A subset of these were analyzed and all had lost their large plasmid. Van Larebeke et al. found a 100% correlation between loss of the plasmid and loss of virulence. Watson et al. (193) demonstrated that the plasmid of avirulent isolates had been lost rather than integrated into the chromosome. In hybridization experiments, the total DNA from cured isolates had no effect on the renaturation kinetics of Ti plasmid DNA purified from the tumorigenic parent strain. This indicated a lack of plasmid-homologous DNA in the cured strains (193).

Following the discovery of Ti plasmids, researchers in the laboratories of J. Schell and E. Nester established that functions for virulence, opine synthesis, and opine catabolism were all encoded on these genetic elements (16, 40, 188, 193, see 84 for review). When octopine- or nopaline-type strains were cured of their large plasmid, they also lost the ability to catabolize opines and the ability to cause tumors. Transfer in planta of the plasmid from virulent donors to avirulent
recipients was accompanied by acquisition of virulence and opine catabolism traits characteristic of the donor. That is, when an octopine-type Ti plasmid was transferred into a cured nopaline-type bacterium, the progeny were found to catabolize octopine, not nopaline. The newly virulent recipients were also found to induce tumors containing the same donor-characteristic opines, even when the donor was an octopine strain and the recipient a cured nopaline-type.

While Ti plasmid transfer into avirulent strains of Agrobacterium was shown to occur readily in planta by Kerr (97, 98) as described above, transfer of the Ti plasmid in vitro was difficult to demonstrate. Subsequently, however, Kerr et al. (103) and Genetello et al. (64) showed that incubation with the opine octopine prior to mating caused octopine-type strains to efficiently transfer their Ti plasmids to recipient strains in vitro. This was facilitated by the discovery, detailed above, that these Ti plasmids encode octopine catabolism, so that incorporation of the opine into the medium could be used to select for transfer of the Ti plasmid. Donors and antibiotic-resistant plasmidless recipients were mixed on filters and incubated on either rich medium plates, or minimal medium plates containing octopine. The cells were then resuspended and spread on selective plates containing antibiotics, glucose, and octopine. The numbers of putative transconjugants appearing were 104-fold higher in matings where the cells had been preincubated on octopine-containing media, than in matings preincubated on rich media lacking octopine. The isolates from matings preincubated on octopine media had acquired the Ti plasmids of the donors. This showed that octopine could serve as a conjugal transfer inducer as well as a nutrient source (64, 103, 142).
In 1977, transfer of virulence from strain to strain had also been demonstrated for the virulence factors of *A. rhizogenes* strains *in planta* (2, 130). By this time, it was widely accepted that these extrachromosomal elements, Ti plasmids in *A. tumefaciens* and the Ri plasmids in *A. rhizogenes* (25), were in fact the virulence factors for these pathogens.

Since *A. rhizogenes* Ri plasmids can be transferred *in planta*, an as yet undiscovered class of opines may serve as their conjugal inducers. Petit et al. (138) showed that *A. rhizogenes* strains A4 and 15834 transfer their plasmids *in vitro* into a cured *A. tumefaciens* strain, in the presence of any one of the mannityl opines (see below). Since the mating and selection steps were performed only on opine-containing media in these experiments, it is not possible to determine whether these opines are inducing conjugal transfer.

In an effort to unify all these data, J. Schell, A. Kerr, and J. Tempé formulated the Opine Concept and the Genetic Colonization scheme (68, 140, 150, 151, 177), hypotheses into which all these original observations, and data collected since, fit remarkably well. According to these two concepts, *Agrobacterium* engineers its own ecological niche by inducing plant tumors that synthesize a unique nutritional source, the opines. The agrobacteria catabolize the opines by virtue of the large Ti plasmid they contain, which is also the active or tumor-inducing principle originally postulated by Braun (18). In addition to serving as a nutritional source, the opines induce transfer of the Ti plasmid to other agrobacteria, which can now take advantage of the opines in the tumor, or induce their own galls.
The existence of the null-type strains posed a problem for proponents of the Opine Concept, as these strains induced tumors that contained no known opines. Nor could most of these strains catabolize octopine or nopaline. Relying on the validity of the hypothesis, researchers looked for previously unidentified opines in tumors induced by null-type strains and discovered a new opine, agropine (59). This was found to belong to a class of four opines, the mannityl opines (35), synthesized in large quantities by tumors induced by these strains. The null-type strains were therefore renamed agropine-type strains.

To date, over twenty different opines have been discovered, comprising several structural categories (Table 1 this work, refs. 13, 21, 26, 35, 38, 49, 50, 59, 66, 67, 90, 95, 172, for review, see ref. 176). The majority of opine classes consist of amino acids linked via an imino bridge to either sugars or carboxylic acids. The agrocinopines, which will be discussed in more detail below, constitute the exception. These opines are sugars joined by phosphodiester linkages. In most cases, a subset of the opines found in the tumor of a particular strain acts to induce conjugal transfer of the Ti plasmid (47, 64). These conjugal opines are usually found in the tumor in lower amounts than the others, which are called the nutritional opines (178).

The Opine Concept has been extended to include A. rhizogenes strains, causative agents of hairy root disease in plants (138). The mannityl and agrocinopine classes of opines, and two new opines, cucumopine (38) and mikimopine (90), have been found in hairy roots induced by A. rhizogenes strains. Some of these strains contain three plasmids, the Ri plasmid, a nononcogenic plasmid, termed an Ar plasmid,
Table 1. *Agrobacterium* strains and the opines found in the neoplasias they induce.

**Representative Strains**

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<tr>
<th>Strains</th>
<th>Opine class</th>
<th>Opines produced</th>
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<tbody>
<tr>
<td>A. <em>tumefaciens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58, T37, K27</td>
<td>Nopaline</td>
<td>Nopaline, nopalinic acid, agrocinopines A + B</td>
</tr>
<tr>
<td>B6, A6, R10, 15955, Ach5</td>
<td>Octopine</td>
<td>Octopine, octopinic acid, lysopine, histopine, mannopine, mannopinic acid, agropine, agropinic acid</td>
</tr>
<tr>
<td>Bo542, A281</td>
<td>Agropine</td>
<td>Agropine, agropinic acid, mannopine, mannopinic acid, leucinopine, agrocinopines C + D</td>
</tr>
<tr>
<td>Eu6, AT181</td>
<td>Succinamopine</td>
<td>Succinamopine</td>
</tr>
<tr>
<td>T10/73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K305, K374</td>
<td>Cucumopine</td>
<td>Cucumopine</td>
</tr>
<tr>
<td>S-4, Sz-1, Sz-2</td>
<td>Vitopine</td>
<td>Vitopine</td>
</tr>
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<td>Strains</td>
<td>Opine class</td>
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<td>A. rhizogenes</td>
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<tr>
<td>A4, 15834,</td>
<td>Agropine</td>
<td>Agropine, agropinic acid, mannopine, mannopinic acid, agrocinopines A + B</td>
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<td>Mannopine</td>
<td>Mannopine, mannopinic acid, agropinic acid, agrocinopines C + D</td>
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<td>TR101</td>
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<td>NCPPB2629,</td>
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<td>NCPPB2659</td>
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<td>NIAE1724</td>
<td>Mikimopine</td>
<td>Mikimopine</td>
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and a cointegrate of the two. In an interesting variation on the
catabolic gene arrangement in Ti plasmids, the genes for opine catabolism
are located both on the Ri plasmids and the nononcogenic Ar plasmids,
instead of on a single virulence plasmid. For example, in agropine-type
strain A4, pRiA4 encodes agropine catabolism (Agr) as well as virulence,
while pArA4a encodes catabolism of the other three mannityl opines;
mannopine (Mop), mannopinic acid (Moa), and agropinic acid (Aga, 138).
The mannopine-type strain 8196 utilizes Mop and Moa well, Aga poorly,
and Agr not at all. pRi8196 encodes the genes for catabolism of two of
these opines, Mop and Moa (138).

There appear to be other plant-microbe interactions that are
analogous to the Opine Concept. There is evidence that the Opine Concept
may be extented to include the genus Rhizobium, which would broaden the
theory to encompass symbiotic, as well as parasitic, relationships. A
compound, L-3-O-methyl-scyllo-inosamine, given the trivial name rhizopine,
has been found in nodules induced by a strain of Rhizobium meliloti (132,
179). This compound is specifically catabolized by the rhizobia by virtue
of genes located on a large plasmid, called the Sym plasmid, which also
encodes nodulation and nitrogen fixation genes. The rhizopine
biosynthetic genes are also found on the Sym plasmid and are closely
linked with the catabolic locus (132). Presumably, rhizopine synthesis
by the bacteroids within the nodules provides a competetive advantage for
rhizopine-catabolizing rhizobia in the soil around the nodule. However,
this selective advantage has not yet been demonstrated.

In an attempt to extend the Opine Concept to other plant-microbe
interactions, Tepfer et al. (181) discovered another interesting
relationship with similarities to the Opine Concept, involving one strain of Rhizobium, *R. meliloti* 41. Only this single isolate, of 42 analyzed, can catabolize bicyclic nitrogenous compounds called calystegins, synthesized by the roots of just three of 105 plant species tested. Calystegin catabolism is plasmid-encoded and independent of nitrogen-fixing symbiosis. Calystegins are abundantly synthesized mainly in the roots of plants such as *Calystegium sepium* (morning glory). Calystegin synthesis differs from rhizopine synthesis in that the former is independent of the presence of rhizobia. The rhizospheres of calystegin-synthesizing plants appear to be enriched for calystegin-catabolizing bacteria compared with the rhizospheres of plants not synthesizing calystegins. This implies a selective advantage for these microorganisms in a natural setting, an aspect of the Opine Concept yet to be tested. Observations such as these indicate that specific exploitation of plant metabolites by bacteria may not be as rare as previously thought. In the cases of calystegins and rhizopines, no evidence has been presented to indicate any transfer of bacterial DNA to the plant.

1.2. The T-region of the Ti plasmid.

Induction of plant neoplasias by agrobacteria and the production therein of opines are important aspects of the Opine Concept. One obvious way of explaining the role of the Ti plasmid in tumor induction and control of opine synthesis was to postulate that the plasmid or a portion thereof is transferred into the plant. This was first suggested by Petit et al. (139) to explain how the bacterial genome determines the opine phenotype of the crown gall tumor. Such transfer was confirmed in both *A. tumefaciens* and *A. rhizogenes* by the demonstration that a segment of
the Ti or Ri plasmid, the T-region, is mobilized into the plant cell. There, it is integrated into the plant cell nuclear DNA, where it is now called the transferred DNA or T-DNA (23, 24, 25, 114, 199).

Several regions of the Ti plasmid are important in this transfer process. The T-region itself is bounded by 25 base pair direct repeats, or borders, which are important in defining the segment to be transferred (190, 204). The other Ti plasmid section important in transfer consists of a series of six operons highly conserved among agrobacteria, the Vir region. The \textit{vir} genes are located within an approximately 35 kb segment of DNA counterclockwise to the T-region. These genes encode functions, inducible by plant-produced compounds such as acetosyringone (166), whose expression is mediated by the products of two of the Vir loci, \textit{virA} and \textit{virC} (168). The remainder of the Vir gene products are thought to process the T-region, starting at the right border. \textit{virD} encodes an endonuclease activity, associated with polypeptides VirD1 and VirD2, which generates nicks in the DNA at the right and left borders, probably interacting with the \textit{virC} gene product (184, 201). Starting at the right border and ending at or near the left, a single-stranded segment of the T-region, the T-strand, is thought to be excised via a strand displacement mechanism (167). Presumably protected by single-stranded binding proteins encoded by \textit{virE} (28), the T-strand is moved into the plant cell. Products of the \textit{virB} region may be involved in transporting the T-strand across the bacterial cell membrane. Several genes in \textit{virB} encode proteins with consensus signal peptide sequences. Furthermore, several proteins encoded by this region are hydrophobic, and are found associated with the cell membrane (54, 183, 192).
Once incorporated into the plant nuclear genome, the T-DNA directs the formation of the plant neoplasia through the overproduction of plant growth hormones. The synthesis of the auxin, indole-3-acetic acid, and the cytokinin, isopentenyladenosine-5-monophosphate, are directed by T-DNA-encoded genes \textit{iaaH} and \textit{iaaM}, and by \textit{iptZ}, respectively (1, 7, 88, 205). There are six transcribed regions, including the three coding for the growth hormone-biosynthetic, oncogenic functions, encoded within the right half of the roughly 22 kb nopaline T-DNA. The octopine T-DNA consists of two segments, T\textsubscript{i} (13 kb) and T\textsubscript{r} (7.8 kb), separated by a central segment of 1.5 kb, T\textsubscript{c} (203). T\textsubscript{i} also contains six transcripts, four of which encode products involved in oncogenesis (113). These display considerable sequence similarity to the nopaline-type onc gene transcripts (92, 198). The oncogenic regions of octopine- and nopaline-type T-DNAs, called the common DNA, are understandably similar, as their functions are the same.

In octopine strains, the T\textsubscript{i} DNA also encodes two genes involved in octopine production; one specifies octopine synthase, the other a function involved in its active secretion (126, 176). Encoded within the right segment of octopine-type T-DNA, T\textsubscript{r}, are three genes involved in the biosynthesis of the mannityl opines (52). These biosynthetic determinants were mapped by demonstrating that T-DNA containing deletions and transposon insertions, when transferred into plants, no longer directs the synthesis of various opines (92, 109). The opine biosynthetic genes for nopaline-type strains are described in the next section. It is not surprising that the opine biosynthetic loci of the two strain types are
unrelated, as they code for the synthesis of completely different sets of opines.

1.3. Nopaline-type strains.

Classical nopaline strains of _A. tumefaciens_ such as C58 induce crown gall tumors that produce nopaline, nopalinic acid, and agrocinopines A and B (Table 1). The genes for the synthesis of nopaline and agrocinopines, nos and acs, are located on the right side and in the center, respectively, of the T-region (92). As described in section 1.1, nopaline, and presumably the agrocinopines, can be catabolized by the inciting bacteria. While the biosynthetic functions are encoded within the T-DNA, determinants for catabolism of these opines are located on the Ti plasmid at 2 o'clock (noc) and 8 o'clock (acc, psc, or agr). Early work mapped these positions by analysis of transposon insertions into and deletions of the Ti plasmid (50, 83).

Nopaline has the classical opine structure, an imino conjugate of an amino acid and an α-carboxylic acid; arginine and α-ketoglutaric acid. The structures for agrocinopines A and B were determined, based on degradative studies, relative electrophoretic mobility profiles and nuclear magnetic resonance analysis of the opines purified from tumors (148). The structures of agrocinopines A and B have recently been confirmed by direct chemical synthesis (60, 115). Agrocinopines differ from the classical opines in that they lack amino acids, and therefore nitrogen. They are complex phosphorylated sugars, the only opine class that contains phosphorus. Agrocinopine A consists of sucrose and L-arabinose linked by a phosphodiester bond from the 2-hydroxyl group of arabinose to the 4-hydroxyl group of the fructose moiety (Fig. 1). The
Figure 1. The structures of agrocin 84 and agrocinopines A and B (134, 145, 148).
AGROCINOPINE A

AGROCINOPINE B

D-GLUCOSE

AGROCIN 84
glucose moiety of agrocinopine A can be removed either enzymatically (α-glucosidase) or chemically, using acetic acid and heat, to produce agrocinopine B (148). It is not known whether both opines are synthesized in the tumor, or whether A is broken down to B. Both compounds are biologically active, inducing conjugal transfer and agrocinopine catabolic functions, as described in sections 1.7 and 1.8.

1.4. Biological control.

Taking advantage of certain susceptibilities of nopaline- and agrocinopine-type A. tumefaciens strains, Kerr (99, 136) has developed a method to control crown gall disease caused by these strains in the field. The key to this method of biological control is a particular strain from a group of nonpathogenic A. radiobacter isolates which have the ability to produce low molecular weight antibiotics called agrocins that act against other agrobacteria. Prior to planting, the roots of plants are dipped in a bacterial suspension of this strain.

A number of agrocin-producing strains have been characterized by Kerr and others, including strains D286 (74), Bo542 (159), J73 (194), K84, K108, and K128 (102). Some agrocin producers, such as J73, Bo542, and K108, are virulent A. tumefaciens strains.

The best studied and the only one of the avirulent strains to be used successfully for the biological control of crown gall as described above is A. radiobacter strain K84 (48, 102, 136). The antibiotic it produces is called agrocin 84, and is a fraudulent adenine nucleoside, containing a 3-deoxyarabinose moiety in place of 2-deoxyribose (ref. 145, Fig. 1 this work). Agrocin 84 is specifically toxic to certain strains of A. radiobacter (144a), A. rhizogenes (2, 130), and A. tumefaciens (102,
Biological control of crown gall with strain K84 is usually successful in those areas where the pathogenic strains are sensitive to agrocin 84. Successes have been reported in Greece (143), Australia (86), Spain (118), Israel (56), Germany (924, Canada, and in the United States (129).

Despite these successes, there have been failures to control crown gall with strain K84. Biological control of crown gall in grapevines has not been successful because most grapevine pathogens are resistant to agrocin 84 (117), and crown gall in grapes involves vascular colonization by the agrobacteria (14). Other failures to control crown gall in the eastern United States (3) and in California (155) also appear to be associated with pathogens resistant to agrocin 84.

1.5. Strain K84.

The nontumorigenic *A. radiobacter* strain K84 has several characteristics that make it uniquely suited to successfully control virulent *Agrobacterium* strains in the rhizosphere. First, production of agrocin 84 by strain K84 is important in its ability to control crown gall. Strains that have lost this ability are less efficient at controlling this disease (32). Second, efficient root colonization appears to be an important factor in biological control by strain K84. Provided the control strain is applied to plants first, some protection can be afforded against agrocin 84-resistant pathogens (31). With the same provision that the control strain is inoculated first, even agrocin-nonproducing (Agr⁻) derivatives of strain K84 still control crown gall, albeit less efficiently (32).
While the nucleoside antibiotic product of strain K84 is specifically toxic to virulent Agrobacterium isolates, strain K84 is, not surprisingly, immune to its action. Production of and immunity to agrocin 84 are both encoded on a 48 kilobase (kb) plasmid of strain K84, pAgK84 (46, 48, 159). The region of pAgK84 encoding agrocin 84 production spans 20 kb. By introducing cloned restriction fragments of pAgK84 in trans to Tn5-induced Agr- derivatives of pAgK84, Ryder et al. (147) found this region to consist of four large complementation groups, suggesting a complex biosynthetic pathway (147). Immunity is conferred by either of two segments mapping within the biosynthetic region. Tn5 insertions into either of these two regions encoded on subcloned restriction fragments abolish immunity to agrocin 84. The same Tn5 insertions carried on pAgK84 eliminate production of agrocin 84, implying that these two processes are associated (147).

Strain K84 contains a second plasmid of 190 kb called pAtK84b. This plasmid encodes catabolism of nopaline and probably agrocinopines A and B (47, 156). Unlike pTiC58, conjugal transfer of pAtK84b is induced by nopaline as well as by agrocinopines (47). Although related to the Ti plasmids, pAtK84b does not encode Vir or T-DNA functions (124, B.G. Clare, A. Kerr, and D.A. Jones, Abstr. 5th Internatl. Congr. Plant Pathol. 1988, 2-47, p. 107).

Armed with the two plasmids pAgK84 and pAtK84b, strain K84 may be viewed as an ecological pirate. This strain catabolizes the opines present in nopaline-type tumors and can colonize plant roots efficiently (99, 156). The strain produces agrocin 84, which has at least in part made it a successful biological control strain for crown gall disease.
Strain K84 has been shown to survive in the rhizosphere while the pathogenic population declines (99). It therefore appears to fit into the Opine Concept relationship as a parasite of a parasite. Strain K84 takes advantage of the niche engineered by certain tumorigenic agrobacteria by catabolizing the opines found in nopaline tumors and eliminating the inciting strains.

1.6. **Agrocin 84 sensitivity and uptake.**

The remarkable specificity of agrocin 84 appears to be an uptake phenomenon; several observations suggest that the antibiotic is transported into sensitive bacteria via a specific Ti plasmid-encoded permease. First, the trait cotransfers with the Ti plasmid (193). Second, deletions of and transposon insertions into one region of the Ti plasmid can abolish agrocin 84 sensitivity (83). Such mutants fail to take up the antibiotic (50). Third, a Ti plasmid-encoded periplasmic protein fraction binds agrocin 84 with high affinity, having a $K_m$ of $5.9 \times 10^{-8}$ M (133). This value is in the same range as those of periplasmic binding proteins associated with transport of other substrates (4).

Agrocin 84 uptake is inhibited in the presence of certain metabolic inhibitors. Incubation with oxidative phosphorylation inhibitors such as sodium azide or dinitrophenol, or with the ATPase inhibitor dicyclohexylcarbodiimide, greatly reduces uptake of $^{32}$P-labeled agrocin 84 by strain K57A (133). Incubation of cells at $0^\circ$C prevents transport of agrocin 84, but returning the temperature to $25^\circ$C results in immediate uptake, indicating that cold treatment does not permanently damage the bacteria, and that a metabolically active cell is a prerequisite for antibiotic transport (133).
Studies on the mode of action of agrocin 84 have shown that it may block attachment of virulent agrobacteria to plant cells (161). Bacterial cells exposed to purified agrocin 84 prior to incubation with cultured tobacco cells bind less efficiently to the plant cells than do untreated bacteria. However, this may just as well be due to toxicity of the agent to the agrobacterial cells. Agrocin 84 also interferes with protein (120) and nucleic acid synthesis (37, 120). The deoxyarabinofuranosyl group of the molecule is structurally equivalent to a dideoxy-nucleoside, and Kerr and Tate (105) have proposed that agrocin 84 has properties making it an ideal DNA chain terminator.

Analysis of the structure of agrocin 84 indicates that the two side groups of the molecule determine specificity and toxicity. Tate et al. (175) showed that removal of the N⁶ glucofuranosyl substituent results in a molecule that is still toxic to agrobacteria at low levels, but the presence of a nopaline-type Ti plasmid is no longer required. This diminished, nonspecific toxicity appears to be a result of reduced uptake of a small amount of the compound diffusing across the cell membrane (134, 175). Removal of the 5' phosphopentanamide group results in loss of toxicity, although the molecule is still specifically transported by strains sensitive to agrocin 84. Isomerization of agrocin 84 so that the 5' group is no longer joined through a phosphoramidate linkage also abolishes toxicity (134, 175). These data imply that the 5' phosphoromidate linkage may be necessary for toxicity. The authors hypothesized that in the absence of a phosphate group, there may not be an appropriate kinase to phosphorylate the nucleoside, a necessary step for formation of the triphosphate and incorporation into DNA (134).
Mutation to agrocin 84 resistance in virulent Agrobacterium strains occurs at frequencies of one in $10^3$ to $10^4$ total viable cells (33). Initial analysis of mutation to agrocin 84 resistance found this to be correlated with the loss of virulence (102) and of the entire Ti plasmid (53). However, Süle and Kado (170) and Cooksey and Moore (33) obtained agrocin 84-resistant mutants that had not lost the Ti plasmid. Both groups isolated mutants of _A. tumefaciens_ strains, including strain C58 and _A. rhizogenes_ strain A4, by plating wild-type cells on minimal solid media containing partially purified agrocin 84. They found that many of these agrocin-resistant mutants had lost their virulence plasmids, or that the plasmids had undergone large deletions. However, both groups obtained agrocin 84-resistant mutants that were still virulent, and displayed no discernable alterations at the restriction fragment level in their Ti plasmid complements. Süle and Kado (170) found that these mutants concomittantly lost the ability to transport labeled agrocin 84. To screen the mutants for reversion to agrocin 84 sensitivity, the authors plated 1000 of them again on minimal medium containing agrocin 84. This revealed a small number of revertants, occuring at a frequency of about one-in-$10^{-3}$, that had become partially sensitive to agrocin 84 (170). The return of agrocin 84 sensitivity was correlated with restoration of antibiotic uptake, implying that these were mutations in the agrocin 84 transport system. All strains were still virulent. These isolates were explained as having undergone frameshift mutations to produce altered but partially active proteins (170).
1.7. *Agrocin 84, agrocinopines, and their transport system.*

The illogic of a system in virulent, sensitive *Agrobacterium* strains for uptake of the toxic compound, agrocin 84, led Ellis and Murphy to search for the true substrates of this transport system. This search ended with the discovery of agrocinopines A and B (50). Using a plate bioassay and analyzing uptake of radioactively labeled agrocin 84, the authors showed that both agrocin 84 sensitivity and transport of this highly specific antiagrobacterial agent are inducible by agrocinopine A. This suggested a link between agrocinopine catabolism and agrocin 84 sensitivity.

Several lines of evidence indicate that agrocin 84 is transported into the cell by the same permease that functions as the first step in the catabolism of agrocinopines A and B. First, Ellis et al. (51) isolated spontaneous transfer-constitutive mutants of pTiC58 (see section 1.9) that are also derepressed for agrocinopine utilization. When incubated with agrocinopine A, one of these mutants removed the opine much more rapidly than did the parent strain C58. The mutants are also supersensitive to agrocin 84. Accumulation of $^{32}$P-labeled agrocin 84 by two such mutants occurred at greatly increased rates compared to strain C58 (51). Second, Ellis and Murphy (50) showed that agrocin 84-resistant mutants no longer transported agrocinopine A. Mutants of the nopaline strains A208 (containing pTiT37) and K57A were isolated by picking colonies growing within the agrocin 84 inhibition zones in plate bioassays. A third mutant, from strain C58 containing a Tn7 insertion into the Ti plasmid, showed a similar block in agrocin 84 transport. All three mutants failed to remove agrocinopine A from culture media (50).
Third, Ellis and Murphy (50) demonstrated that agrocinopine A blocks agrocin 84 transport. Induction of nopaline strain K57A with agrocinopine A prior to exposure to $^{32}$P-labeled agrocin 84 caused an increase in the transport of the antibiotic over the uninduced strain incubated only with labeled agrocin 84. However, when uninduced cells were incubated with $^{32}$P-agrocin 84 and a molar excess of agrocinopine A simultaneously, agrocin 84 transport was completely blocked, presumably by a competitive mechanism. Last, agrocin 84 sensitivity and agrocinopine A and B catabolism functions have been located on the Ti plasmid. Analyzing Tn1 and Tn7 insertions, and transposon-induced deletions of pTiC58, Schell and coworkers (83, 152) and Ellis and Murphy (50) located a region encoding agrocin 84 sensitivity and agrocinopine uptake at about 87 megadaltons (approximately 132 kb) on the Ti plasmid map of Depicker et al. (41). Cooksey isolated a spontaneous agrocin 84 resistant mutant of strain C58 by spreading cells on plates of minimal medium containing agrocin 84, as described above. It was found to have an indigenous insertion sequence in this region (30). The insertion sequence is similar in sequence to chromosomal DNAs of several virulent strains, but not to the wild-type Ti plasmid. Cooksey postulated that the sequence may be responsible for the deletions frequently observed in the Ti plasmids of agrocin 84-resistant mutants (30). Although these analyses have provided crude mapping data, the precise location of genes encoding the agrocinopine-agrocin 84 transport system and their position relative to catabolic determinants has not yet been elucidated.

Structurally similar regions of the lower faces of the agrocinopine arabinose moiety and the agrocin 84 N6 glucosyl substituent (Fig. 1) may
be recognized by a common transport system. This would be consistent with the agrocin 84 structure-function studies of Murphy et al. (134), and with the observation that both agrocinopine A and agrocinopine B are biologically active as inducers of agrocin 84 sensitivity and conjugal transfer (47, 50). Nevertheless the formal possibility still exists that there are separate permeases for agrocin 84 and the agrocinopines. Whether an agrocinopine catabolic pathway is associated with uptake of the three compounds, and any relationship of such a pathway to agrocin 84 sensitivity is also unknown. Strain K84 appears to have developed a biosynthetic pathway for an antibiotic structurally quite different from the agrocinopines (Fig. 1), but similar enough to use the same transport system; a molecular Trojan horse.

1.8. Agrocinopines and conjugal transfer.

The agrocinopines are interesting in that they apparently share their transport system with an antibiotic. A second important characteristic of these opines is that they function as the inducers of conjugal transfer of the Ti plasmid for nopaline-type Agrobacterium strains. They are instrumental in the dissemination of the Ti plasmid among agrobacteria.

Ellis et al. (47) showed that growth of A. tumefaciens strains C58 and T37 in the presence of agrocinopine A or B increases the frequency at which they transfer their Ti plasmids by about four orders of magnitude. Interestingly, agrocinopines induce the conjugal transfer of pAtK84b, the large opine catabolic plasmid of A. radiobacter strain K84 (47). Nopaline will also act as a conjugal transfer inducer of pAtK84b, but not for pTiC58 or pTiT37 (47). Ellis et al. also showed that incubation of strain
C58 with agrocinopine A increased the rate of opine uptake by this isolate (51). Agrocinopine A therefore induces agrocinopine transport, which is the first step of catabolism, and both opines induce pTiC58 for conjugal transfer; these two plant-synthesized compounds are the conjugal opines for pTiC58.

Induction by plant products is a feature shared by conjugal transfer of the Ti plasmid from one bacterium to another and transfer of the T-DNA from an agrobacterial cell into a plant cell. The two processes share other features as well. As described in section 1.2, T-DNA transfer is thought to involve a nick generated at the right border of the T-region, followed by removal of the single-stranded T-strand by a strand displacement mechanism (167). Conjugation of the fertility factor F of E. coli, the model system for conjugation in Agrobacterium, proceeds in a similar fashion. A nick is introduced at oriT, and one strand is transferred to the recipient, while the donor synthesizes a replacement strand (197). The products of virD1 and virD2, which introduce the nick in the Ti plasmid, have their analogues in the F system, traY, traI, and traZ. The transferred strand of F is thought to be coated with a single-stranded binding protein (197), much like the virE product and the T-strand.

Although there is evidence that T-DNA processing and conjugal transfer are independent processes (8), some of their components can apparently be interchanged. Buchanan-Wollaston et al. (19) found that a plasmid derivative containing the oriT of the wide-host-range plasmid pRSF1010 and mob functions, which nick the pRSF1010 oriT site, can be transferred and stably incorporated into the genome of plant cells. The
plasmid was transferred only when Ti plasmid vir genes were supplied in trans. Thus, oriT of pRSF1010, with its mob nicking function, can substitute for a proper T-DNA border when one is not present. Not only does this indicate that conjugal plasmid and T-DNA transfer functions are analogous, it also implies that plants may have access to a large bacterial gene pool.

1.9. Coregulation of opine catabolism and conjugal transfer.

There are two cornerstones of the Opine Concept. The first is the ability of virulent agrobacteria to catabolize the opines in the tumors they cause. The second is the conjugal inducer characteristic of some opines. This allows agrobacteria to transfer their Ti plasmid, and consequently its tumor-inducing ability, among themselves. A disseminated Ti plasmid would generate an increased tumor-inducing potential, and with it the possibility of an increased opine supply. Negative coregulation of these two functions, conjugal transfer and opine catabolism, with the opines themselves as inducers, would allow the fullest exploitation of this ecological niche. With this type of regulation, opine catabolic functions, including those for transport, would not be wastefully synthesized unless opines were present. Similarly, the energy-intensive process of Ti plasmid transfer would not occur unless the donor was in the neighborhood of an opine-synthesizing tumor, where the opine catabolic and tumorigenic traits of the plasmid would be of the most benefit.

Studying octopine-type strains R10 and Ach5, respectively, Petit et al. (141) and Klapwijk et al. (106, 107) both proposed that octopine catabolism (Occ) and conjugal transfer (Tra) of the octopine Ti plasmids were coregulated by a common repressor. The noninducing octopine analog,
noroctopine was used to select spontaneous mutants constitutive for octopine catabolism (Occ\(^c\)). When assayed for conjugal transfer, both groups found that some of the mutants were constitutive for conjugal transfer as well. Petit et al. (141) isolated three classes of noroctopine-selected Occ mutants. The mutants included isolates constitutive for both Occ and Tra (Tra\(^c\)), mutants with Occ and Tra phenotypes inducible by both octopine and noroctopine, and strains exhibiting a range of constitutive phenotypes for both characteristics. Klapwijk et al. (106) isolated the first two classes of mutants in octopine-type strain Ach5. They also isolated Occ\(^c\) mutants whose Tra phenotype was still octopine-inducible, and Tra\(^c\) strains whose Occ phenotype was still inducible by octopine. They postulated that those strains in which only one of the two phenotypes was constitutive resulted from operator mutations. To explain the mutant class whose catabolic and Tra phenotypes were noroctopine-inducible, these workers postulated a regulatory gene mutated so that its product now recognized the analog as an inducing substrate. Both groups of workers suggested that the simplest interpretation of the data was one in which a single repressor regulates both tra and occ.

Regulation of these two regions appears to be more complex. When Occ\(^c\) mutants were plated on medium containing the toxic analog homooctopine, mutants that were unable to catabolize octopine were isolated. When tested for transfer of their Ti plasmid, some of these Occ\(^-\) isolates were found to be Tra\(^-\) as well. A portion of these were revertible, suggesting a point mutation in a regulatory function. Klapwijk et al. (106) suggested that these had been mutated in a factor
required for expression of both phenotypes, such as a shared operator region, an activator, or a regulatory function having positive and negative effects. Another possible explanation for the Occ⁻Tra⁻ phenotype was superrepression, or the reduction of gene expression levels to an undetectable level by a strong negative regulator. It should be possible to overcome superrepression by addition of excess inducer, but incubation of these revertible Occ⁻Tra⁻ mutants with high levels of octopine had no effect on the two mutant phenotypes (106).

In order to establish dominant and recessive relationships for tra and occ functions, Klapwijk et al. (107) constructed a plasmid containing the occ and tra regions of octopine Ti plasmid pTiB6S3 cointegrated with a replicon compatible with octopine Ti plasmids. This allowed construction of merodiploids and analyses of functions provided in trans. These merodiploids also harbored Ti plasmids containing antibiotic resistance markers, allowing their transfer to be followed. The recombinant plasmid, pALl16, was introduced into strains containing marked Ti plasmids with the following phenotypes: Occ⁺ Tra-inducible (Tra⁺), Occ-inducible (Occ⁺) Tra⁺, Occ⁺ Tra⁺, and Occ⁻Tra⁻. Introduction of pALl16 into the Occ⁺ Tra⁺ strain resulted in an Occ⁺ Tra⁺ phenotype, consistent with the presence of a negative regulator acting in trans. The presence of pALl16 in the Occ⁺ Tra⁺ mutant did not alter either phenotype. This supports the proposal of Klapwijk et al. (107) that the Tra⁺ phenotype is a result of an operator mutation, so long as one assumes a single regulated Tra region. In apparent contradiction to the above proposal was the discovery that introduction of pALl16 into an Occ⁺ Tra⁺ resulted in an Occ⁺ phenotype, again indicating that regulation of this region is probably more complex.
than the model predicts. According to the model, an Occ\textsuperscript{c} Tra\textsuperscript{1} phenotype resulted from a mutation in the occ operator, and a negative regulator supplied in trans should have had no effect on this postulated cis-acting mutation. The authors of this article ruled out superrepression as an explanation for the Occ\textsuperscript{-}Tra\textsuperscript{-} strains by introducing pAL116 and showing that the resulting strain is Occ\textsuperscript{+}. They did not report the transfer phenotype of this strain, which would have been helpful in determining the existence of an activating function.

In strain C58, pTiC58 conjugal transfer and agrocinopine catabolism functions are both induced by one group of opines, agrocinopines A and B (51). A similar situation exists with the agropine-type Ti plasmids and agrocinopines C and D (47). This suggests that the two operations, conjugal transfer and agrocinopine catabolism, are coordinately regulated by the agrocinopines, the conjugal opines, in a manner analogous to that of the octopine-type Ti plasmid. In their study of conjugal transfer of pTiC58, Ellis et al. (51) isolated and analysed a series of transfer-constitutive mutants of strain C58. These mutants were selected by spreading Ti plasmid-containing donor and Ti plasmidless recipient cells on minimal medium plates containing nopaline as the sole carbon and nitrogen source. The rare colonies that arose were retested in matings to determine whether the Ti plasmid had mutated to confer a Tra\textsuperscript{c} phenotype. One of these mutants, C58C1ChlEry(pWI1000), is the Tra\textsuperscript{c} strain referred to as K439 in this work. In addition to becoming Tra\textsuperscript{c}, this strain concurrently acquired three other phenotypes. First, it removes agrocinopine A from culture media much more rapidly than its wild-type parent, C58 (51). Second, this strain and another Tra\textsuperscript{c} isolate,
C58G1Ch1Ery(pWI1009), are both supersensitive to agrocin 84, and third, both mutants take up the radioactively-labeled antibiotic much more rapidly than does the parent strain. These strains have simultaneously become constitutive for conjugal transfer, for agrocin 84 and agrocinopine uptake, and for agrocin 84 sensitivity.

An exception to these observations was an isolate containing the Tra⁺ plasmid pWI1003. Instead of showing supersensitivity to agrocin 84, this strain is resistant to the antibiotic. Ellis et al. (51) did not determine whether plasmid pWI1003 was the product of two mutations, one resulting in a Tra⁺ phenotype and the other creating an agrocin 84-resistant phenotype, or if the two phenotypes were caused by a single mutation. From their observations, Ellis et al. have proposed that the defect in the Tra⁺ agrocin 84-supersensitive strains may affect a single repressor function governing conjugal transfer and uptake of both agrocin 84 and the sugar opine. This is consistent with the observations that strains such as C58 and A208 (156), containing wild-type Ti plasmids, while sensitive to agrocin 84, can be made supersensitive by prior exposure to agrocinopine A (50, see sections 1.7 and 1.8). Strains K439 and LBA4011(pTiC58Tra⁺) (51, 93), both of which are presumably repressor mutants of strain C58, are supersensitive to agrocin 84 and constitutive for agrocinopine uptake and conjugal transfer.

1.10. Agrocin 84 secretion.

Ellis et al. (51) noted that after rapidly accumulating radioactively labeled agrocin 84, the counts associated with their Tra⁺ mutants begin to drop. They postulated that this could be due to leakage of agrocin 84 or its breakdown products from the cells. To determine
whether their Tra<sup>c</sup> plasmids encoded the breakdown of agrocin 84, they tested these elements in strains containing the agrocinogenic plasmid, pAgK84, for agrocin 84 secretion. With one exception, all of the strains containing pAgK84 and different Tra<sup>c</sup> plasmids failed to produce any detectable toxic substance when tested in the agrocin 84 plate bioassay. The only strains that produced an inhibitory compound were those containing pAgK84 and either pWI1003 or pTiC58.

The correlation between a constitutively expressed agrocinopine-agrocin 84 region and failure to secrete detectable amounts of agrocin 84 by Tra<sup>c</sup> strains containing pAgK84 led the authors to suggest that Tra<sup>c</sup> strains broke down agrocin 84. This accounted for the loss of radioactive counts when strains containing only a Tra<sup>c</sup> Ti plasmid took up labeled agrocin 84. The breakdown products were presumed to remain toxic, since Tra<sup>c</sup> strains are supersensitive to agrocin 84. It was thought possible that the breakdown products were secreted, but were not detected because they were no longer specifically taken up (51). These presumptions are not necessarily in conflict with the work of Murphy and Roberts (133), who analyzed uptake of radioactively-labeled agrocin 84 by pTiK27-containing strain K57A. They demonstrated that this strain takes up the antibiotic, reaching a maximum level of accumulated counts per min at two h, which then falls off by approximately 25% by four h. Ellis et al. (51) made an alternative proposal to agrocin 84 breakdown to explain the lack of agrocin 84 secretion by pTiC58Tra<sup>c</sup>, pAgK84-containing strains. They suggested that the agrocin 84 was reaccumulated by the derepressed (constitutively expressed) Ti plasmid-encoded permease as fast as it was secreted.
1.11. **Agrocinopine-agrocin 84 regions in other Agrobacterium strains.**

Classical *A. tumefaciens* nopaline strains include C58, T37 and K57A. All elicit tumors that contain agrocinopines A and B, and in each, the Ti plasmid encodes agrocinopine A-inducible sensitivity to agrocin 84 (50). However, agrocin 84 sensitivity is not limited to these classical nopaline-type strains. *Agrobacterium rhizogenes* strains A4 and 15834 are sensitive to agrocin 84 and also induce neoplastic growths apparently containing agrocinopines (50, 130, 138, 196). This is true as well, with qualifications, for strain A281, which contains the agropine-type Ti plasmid, pTiBo542 (50). Tumors induced by strains harboring pTiBo542 synthesize the uncharacterized sugar opines agrocinopines C and D, and agrocin 84 sensitivity of strain A281 is only expressed in the presence of these opines (50). Furthermore, strain J73, a tumorigenic nopaline-type strain isolated in South Africa, which produces its own agrocin, is sensitive to agrocin 84 (194). Kerr has isolated *A. radiobacter* strains that, although avirulent, are sensitive to agrocin 84 and can catabolize the opines found in nopaline tumors (104). From the evidence cited in section 1.7, agrocinopines A and B, and agrocin 84 appear to be taken up by a common transport system in the classical nopaline-type *A. tumefaciens* strains. It seems likely that other *Agrobacterium* strains sensitive to this same antibiotic and inducing the synthesis of the same or similar opines in plants would contain related regions. These similarities should be detectable at the DNA sequence level.

1.12. **Purpose of this work.**

A number of questions remain to be answered concerning the region encoding agrocin 84 sensitivity and agrocinopine catabolism. What is the
genetic organization of this region on pTiC58? Is there only one transport system for both agrocin 84 and agrocinopines? How is the region regulated? How many functions does it encode and what are they? How closely is it related to other similar regions? To answer these questions, we have cloned the agrocinopine-agrocin 84 region. In this study we have analysed the phenotypes conferred on _A. tumefaciens_ strains containing these clones. In addition, we have used subcloning and transposon mutagenesis to map the region and to demonstrate that its expression is regulated by the agrocinopine opines. To determine the genetic organization and encoded protein products of the _acc_ region, complementation experiments, maxicell analyses and catabolism studies were performed. Southern hybridizations were used to determine the extent of relatedness between this region and other _Agrobacterium_ plasmids conferring similar phenotypes. To ascertain the functional extent of the similarities between the _acc_ regions of pTiC58 and pAtK84b a comparison of these two plasmids was further pursued with respect to agrocin 84 sensitivity, and agrocinopine and agrocin 84 transport.
2.1. Bacterial strains and plasmids.

Strains and plasmids used in this study are listed in Table 2. Agrobacterium strains were grown at 28°C, and Escherichia coli strains were grown at 37°C. Liquid cultures were grown on rotary shakers at 150 rpm. Small overnight cultures (1.5 to 2 ml) were grown in loosely-capped 12 x 1.6 or 15 x 1.6 cm glass screw-cap tubes (Kimble, Owens-Illinois, Toledo, OH), and larger cultures were grown in 125 ml (20 ml cultures) or 1000 ml (200-250 ml cultures) erlenmeyer culture flasks (Bellco Glass, Inc., Vineland, NJ). Cosmid clones and subclones were isolated in E. coli strains DH1 and HB101 (119), Tn2-HoHol derivatives were isolated in E. coli strain C2110 (165), and Tn5 derivatives were isolated in E. coli strain HB101 and A. tumefaciens strain C58ClRS. In earlier experiments involving a conjugal transfer-constitutive (Tra+) mutant of strain C58, strain K439 was used. The Ti plasmid of this strain was found to have rearrangements and deletions when restriction digestions of plasmid DNAs from strains K439 and C58 were compared. The Ti plasmid of strain LBA4011(pTiC58Tra+) (29, 93) was chosen to replace it for subsequent experiments. The restriction patterns for pTiC58Tra+ and pTiC58 are indistinguishable (S. Von Bodman, personal communication). None of the rearrangements or deletions of the Ti plasmid from strain K439 map to the region containing acc, however, and all examined acc-associated phenotypes in strains K439 and NT1(pTiC58Tra+) are indistinguishable. Likewise, merodiploid Agrobacterium strains were first constructed using strain NT1.
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<tr>
<td>pTHH352</td>
<td>(pTHH206::Tn5)</td>
<td>Agr84 Cm Km</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Continued

Abbreviations: Acp, agrocinopine transport; Acs, agrocinopine synthesis in tumors; Aga, agropinic acid catabolism; Agr, agropine catabolism; Agr73\(^+\), agrocin 73 production; Agr73\(^-\), agrocin 73 sensitivity; Agr84\(^-\), agrocin 84 immunity; Agr84\(^+\), agrocin 84 production; Agr84\(^\Delta\), agrocin 84 resistance; Agr84\(^\gamma\), agrocin 84 sensitivity; Agr84\(^\gamma\)\(^\gamma\), agrocin 84 supersensitivity; \(\beta\)-Gal, \(\beta\)-galactosidase; Gly\(^-\), glycine auxotrophy; leu, leucine auxotrophy; Met\(^-\), methionine auxotrophy; Moa, mannopinic acid catabolism; Mop, mannopine catabolism; Pro\(^-\), proline auxotrophy; ser, serine auxotrophy; thi, thiamine auxotrophy; IncPl, incompatibility group Pl; IncQ, incompatibility group Q; IncW, incompatibility group W; Noc, nopaline catabolism; pol, DNA polymerase I; rec, recombination; Tra\(^C\), constitutive conjugal transfer; Tra\(^\Delta\), inducible conjugal transfer; Ap, ampicillin resistance; Cb, carbenicillin resistance; Cm, chloramphenicol resistance; Em, erythromycin resistance; Gm, gentamicin resistance; Km, kanamycin resistance; Nm, neomycin resistance; Nx, nalidixic acid resistance; Rm, rifampicin resistance; Sm, streptomycin resistance; Sp, spectinomycin resistance; Tc, tetracycline resistance.
Upon its construction, the recA erythromycin-resistant (Em') NT1 derivative, UIA143 (57), was used to replace strain NT1 in creating merodiploid strains. Vector pSa4ΔH was constructed by digestion of pSa4 (173) with HindIII and religation, eliminating three small HindIII fragments of pSa4 (J. Slota, personal communication). This plasmid confers resistance to chloramphenicol and kanamycin on Agrobacterium. While E. coli strain HB101 containing pSa4ΔH grows well on NA containing 25 µg/ml chloramphenicol, the strain grows poorly on NA plus kanamycin even at the lowered concentration of 10 µg/ml.

2.2. Media.

Media used were L broth (LB, Gibco Laboratories, Madison, WI), Mueller Hinton agar (MH, Difco Laboratories, Detroit, MI), Nutrient Agar (NA, Difco), Stonier's medium (169), AB minimal medium (22) and AT medium (180). Agar (Difco) was added at 1.5% to L broth (LA), Stonier's medium and AB minimal medium for solid media. Glucose was used as a carbon source at 0.5% in AB medium. (NH₄)₂SO₄ was provided in AT medium at 0.15% (ATN). Minimal medium for E. coli strains (EC) contained 3.0 g K₂HPO₄, 1.0 g KH₂PO₄, 5.0 g NH₄NO₃, 1.0 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O and 20 g glucose per liter. Tryptone yeast extract (TYE; 191), yeast mannitol agar (YMA; 162), and yeast extract broth (YEB; 142) were used to grow A. rhizogenes strains. Beef extract, peptone, tryptone, and yeast extract used in the A. rhizogenes media were from Difco. M63 medium containing 0.4% glucose and 20 µg/ml methionine (158) was used for growth of the maxicell strain LCD44. In selective media for E. coli, tetracycline was used at 10 µg/ml, ampicillin at 60 µg/ml, chloramphenicol at 25 µg/ml, gentamicin at 50 µg/ml, kanamycin at 50 µg/ml, nalidixic acid at 4 µg/ml, neomycin at 150
µg/ml, and spectinomycin at 150 µg/ml. For Agrobacterium, carbenicillin
was used at 50 or 100 µg/ml in place of ampicillin, chloramphenicol at 25
µg/ml, erythromycin at 150 µg/ml, gentamicin at 50 µg/ml, kanamycin at
50 µg/ml, neomycin at 150 µg/ml, rifampicin at 50 µg/ml, streptomycin at
200 µg/ml and tetracycline at 1 or 2 µg/ml.

2.3. Chemicals.

All chemicals were from Sigma Chemical Co., St. Louis, MO unless
otherwise noted. Cesium chloride was from Boehringer Mannheim
Biochemicals, Indianapolis, IN. Pyridine was from Anachemia, Champlain,
NY. Dimethyl formamide, isoamyl alcohol, phenol crystals, sodium
carbonate, and sulfuric acid were from J.T. Baker Chemical Co.,
Phillipsburg, NJ. Acrylamide, ammonium persulfate, N,N'-methylene-bis-
acrylamide, bromphenol blue (BPB), sodium dodecyl sulfate (SDS) for
electrophoresis, and N,N,N',N'-tetramethylethylenediamine (TEMED) were
from Bio-Rad Laboratories, Richmond, CA. Chloroform, dimethyl sulfoxide
(DMSO), glycerol, isopropanol, potassium phosphate, and sodium sarkosyl,
and toluene were from Fisher Scientific, Fair Lawn, NJ. Silver nitrate,
xylene cyanole, GBX and Dektol developer, and fixer were from Eastman
Kodak Co., Rochester, NY. Agarose (HGT) was from Seakem™, FMC
Bioproducts, Rockland, ME. Ammonium chloride, 1-butanol, calcium
chloride, concentrated HCl, 2,5-diphenyloxazole (PPO), ferrous sulfate,
glacial acetic acid, magnesium chloride, magnesium sulfate, Orange G,
potassium chloride, sodium chloride, sodium hydroxide, and sodium
phosphate were from Mallinkrodt Chemical Works, Paris, KY. Ethanol (100%,
reagent grade) was from USI Chemicals Co., Tuscola, IL.
Partially purified preparations of agrocinopines A and B and agrocin 84 were the generous gifts of M. Ryder. The sugar phosphate opines are generally found as a mixture in tumors induced by nopaline strains of Agrobacterium (148). The two can be separated (148), but both opines induce conjugal transfer and agrocin 84 sensitivity in the nopaline strain C58 (47, 50). Consequently, we made no effort to further resolve the mixture, which is hereafter referred to as agrocinopines A+B. Agrocinopine concentrations, expressed in arabinose equivalents, were determined using the phloroglucinol reagent of Dische and Borenfreund (44) with arabinose (Sigma) as the standard. To perform the phloroglucinol assay, opine or arabinose samples were brought up to 200 µl with distilled water, then mixed in 100 X 7.5 mm borosilicate glass tubes with 2.5 ml phloroglucinol reagent [0.22 % (w/v) phloroglucinol, 0.71 % (w/v) glucose (optional), 97.3 % (v/v) glacial acetic acid, 1.8 % (v/v) concentrated HCl]. The tubes were boiled vigorously for 15 min in a water bath and cooled in tap water. The $A_{512}$ and $A_{552}$ were measured using a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, NY). The former reading was subtracted from the latter to give a value proportional to the arabinose concentration of the sample. Standard curves plotting amounts of arabinose (µg) versus the difference between $A_{552}$ and $A_{512}$ were constructed using 10, 20, 50, 100, 150, and 200 µl of an arabinose stock solution (25 mg/ml) diluted 1:100 prior to use.

In initial experiments to produce agrocinopine B sufficiently pure for use as a sole source of carbon and energy, agrocinopine A (approx. 10 mg/ml) was converted to agrocinopine B and glucose by boiling it in 1.5 M acetic acid for 15 min. The reaction products were separated by
preparative high voltage paper electrophoresis (HVPE) and the agrocinopine B was eluted from the paper as described later in this section. To remove contaminants catabolizable by bacteria lacking a Ti plasmid, the eluted opine was biologically purified as follows. The opine preparation (400 µl) was mixed with an equal volume of 2 X ATN, inoculated with strain NT1, and incubated at 28°C for 5 to 7 days. The cells were then removed by centrifugation in a Fisher Model 235B microcentrifuge for 5 min. The agrocinopine B in the supernatants were further purified by a method patterned after that of Ryder et al. (148). To sterilize the culture medium, a portion of a tissue (Kimwipes™, Kimberley-Clark Corp., Roswell, GA) was crumpled to fit into a 1.5 ml microcentrifuge tube, wetted with chloroform, inserted into such a tube containing the culture supernatant, and allowed to stand in a flow hood for ten min. The tissue was removed and the tube left in the hood for five min. The chloroform-sterilized culture medium containing the agrocinopine B was applied to a 1.5 ml column of Dowex AG1-X2 (acetate form) in a Pasteur pipette. The column was washed with several volumes of distilled water. Initially, agrocinopine B was eluted from ion exchange columns using 10 column volumes of a single buffer concentration (0.2 M pyridine, 0.1 M acetate). Fractions (1 ml) were collected, and phloroglucinol-positive (44) fractions (fractions 2 through 6) were pooled. The pool was applied to a 1.5 ml Dowex 50 (Na⁺ form) column and eluted with distilled water. Phloroglucinol-positive fractions were again collected (fractions 1 and 2), evaporated to dryness, and resuspended in distilled water.

In later experiments, agrocinopine B (300 µl, approx. 5 mg/ml, provided by M. Ryder) isolated from tumors was biologically purified to
remove any other contaminants that Ti-plasmidless agrobacteria can catabolize. After chloroform sterilization and application to a Dowex AG1-X2 column, the opine was eluted with one column volume each of a series of pyridine-acetate buffers increasing in concentration by increments of one tenth of the final concentration, from 20 mM pyridine, 10 mM acetate to 200 mM pyridine, 100 mM acetate. Fractions (1 ml) were collected, and phloroglucinol-positive fractions (fractions 17 through 23) were pooled. These were subjected to Dowex 50 column chromatography as described above, dried and redissolved in distilled water to a concentration of 50 mM agrocinopine B.

For in vitro catabolism experiments, partially purified agrocinopine B (approximately 5 mg/ml, 40 µl) was spotted on Whatman 3 MM paper in a line perpendicular to the direction of electrophoresis and subjected to HVPE in formate/acetate buffer. Edge strips approximately 2 cm wide were cut from the paper and stained with alkaline silver nitrate to locate the agrocinopine B band (see section 2.11 for description of HVPE and staining). The section of the unstained paper containing the opine was cut out and segments (approximately 2 x 5 cm) were rolled up to fit into plastic 0.5 ml microcentrifuge tubes. The lids of the tubes were removed and holes were made in the bottoms with a hot inoculating needle. The tubes were then placed in 1.5 ml plastic microcentrifuge tubes. Samples of distilled water (100 µl) were applied to the rolled papers, and the tube assemblies were centrifuged at room temperature (RT) for 2 min in a Fisher microcentrifuge. The eluted agrocinopines, collected in the bottom of the 1.5 ml tubes, were removed and pooled. This elution process was performed 6 times for each paper segment. The water was evaporated from
the eluted agrocinopines using a Savant Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, NY) and a Welch Duo-Seal vacuum pump (Sargent-Welch Scientific Co., Skokie, IL), and the opines were redissolved in 40 µl distilled water.

2.4. Plasmid isolation.

Plasmids under 50 kilobases (kb) in size were isolated using the alkaline lysis method of Maniatis et al. (119) from either small (1.5 mls) and large (250 mls) cultures. Purification of large (ca. 200 kb) plasmids was performed using the method of Casse et al. (20), or by a method combining elements of the Maniatis and Casse protocols (J.E. Slota and S.K. Farrand, personal communication). Cells grown to late log phase in LB were harvested by centrifugation (4°C) at 10,000 X g in 300 ml polypropylene centrifuge bottles for 15 min using a Sorvall GSA rotor (Du Pont Co., Wilmington, DE). The cell pellet was resuspended in 15 ml washing buffer [50 mM Tris-HCl, 20 mM disodium ethylenediamine tetra-acetic acid (EDTA), 0.5 M NaCl, 0.05% sodium sarkosyl, pH 8.0] and again centrifuged. 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, 1.0% SDS) was added and the bottle contents mixed gently, followed by a 10 min incubation at RT. Lysozyme (2 mg/ml) was added to solution 1 in subsequent purifications if lysis was not complete. Tris-HCl (2 M, pH 7.0, 7.5 ml) was added and the contents thoroughly and gently mixed. NaCl (5 M, 7.5 ml) was added, the contents were mixed gently, and the bottles were left standing for 10 to 20 min at RT. Phenol (60 ml) saturated with 3% NaCl was added and the aqueous phase was extracted for 2 to 5 min by
gentle agitation to form a well-mixed emulsion. The bottles were centrifuged at 10,000 X g for 10 min at 4°C, and the aqueous phases transferred to clean bottles. Ice cold ethanol (95%, two volumes) was added, the bottle contents were mixed and stored at -20°C for 2 h to overnight. The precipitated DNA was collected by centrifugation (4°C) at 10,000 X g for 20 min. The pellets were washed with ice cold 70% ethanol (50 ml), and bottles recentrifuged as above. The ethanol was decanted, the pellets were air dried for about 5 min, and redissolved in TES (30 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) prior to centrifugation in cesium chloride-ethidium bromide gradients.

Plasmid DNA from large cultures was purified by subjecting it to two centrifugations to equilibrium in cesium chloride-ethidium bromide gradients. Cesium chloride (4.3 g), dissolved in 2.0 ml of TES, was mixed with plasmid DNA dissolved in 1.9 ml TES, and 0.3 ml of 10 mg/ml filtered (Nalgene disposable filter unit Type A, Nalge Co., Rochester, NY) ethidium bromide. The solution was transferred by Pasteur pipette into polyallomer 11 X 32 mm Quick-Seal™ tubes (Beckman Instruments, Inc., Palo Alto, CA), which were sealed (Beckman Tube Sealer) and placed in a VTi80 rotor (Beckman). Centrifugation was performed at 60,000 revolutions per minute for 4 to 12 h in an L8-70M Beckman ultracentrifuge. Plasmid bands, visualized with a UVSL.25 hand-held ultraviolet lamp (Ultra-Violet Products, Inc., San Gabriel, CA), were harvested through the tube wall using a 1 cc syringe and an 18G needle (Becton-Dickinson & Co., Rutherford, NJ). Ethidium bromide was removed by repeated extraction of the harvested DNA solution with equal volumes of isopropanol saturated with 20X SSC (3.0 M NaCl, 0.3 M sodium citrate pH 7.0, ref. 119). The
aqueous phase was diluted with LTE (1.5 volumes, 10.0 mM Tris-HCl, 1.0 mM EDTA, pH 8.0), followed by addition of 0.1 volume 5M potassium acetate and two volumes of ice cold 95% ethanol. The DNA was precipitated at -20°C overnight, and collected by centrifugation at 4°C for 30 min at 17,000 X g. The DNA pellets were washed with 5 ml ice cold 70% ethanol followed by centrifugation at 17,000 X g for 20 min at 4°C. The pellets were air dried and redissolved in LTE (approx. 300 µl).

2.5. Restriction endonuclease digestion and agarose gel electrophoresis.

Restriction endonuclease digestion of plasmid DNA was performed as described by Slota and Farrand (159). Restriction endonucleases were from Bethesda Research Laboratories (BRL), Rockville, MD. Electrophoresis was performed horizontally using agarose gels (0.7%, Seachem HGT) and Tris borate EDTA buffer [TBE, 89 mM Tris (Sigma 7-9 was used), 89 mM boric acid, 2 mM Na₂EDTA; refs. 117, 125, 157] was employed. Electrophoresis chambers were from BRL (model HO/H2) and Hoeffer Scientific Instruments, San Francisco, CA (models HE 33 and HE 99). Constant current electrophoresis was performed using PS1200 (Hoeffer), EC452 (E-C Apparatus Corp., St. Petersburg, FL) and 401 (Epigene, Inc., Baltimore, MD) power supplies, at current levels of 2 to 50 mA, until the dye front neared the end of the gel. Tracking dye (TD, 2 µls added per 10 µl electrophoresis sample) contained 33% glycerol, 7% SDS and 0.07% BPB. Gels were stained in deionized water containing approximately 0.5 µg/ml ethidium bromide for at least 30 min prior to photography using a Model TM-36 transilluminator (Ultra-violet Products, Inc., peak wavelength 302 nm), a 15G orange filter, a UV17 ultra-violet filter (Tiffen Manufacturing Corp., Hauppauge, NY), and type 667 or type 55 film (Polaroid Corp., Cambridge, MA).
2.6. **Cosmid cloning and subcloning.**

Cloning into cosmids pVK102 (108) and pCP13 (36) was performed as outlined by Hohn (81) using partial digests of pTiC58 and the bacteriophage $\lambda$ packaging kit from Amersham Corp., Arlington Heights, IL. In a pilot experiment, approximately 0.5 µg amounts of Ti plasmid DNA were separately digested using approximately 10 units of restriction endonucleases EcoRI, BamHI and HindIII in 50 µl reaction volumes. Samples (10 µl) were removed at 1, 2, 4, 8, and 60 min intervals, and the reactions stopped by addition of 2 µl TD containing 0.1 M EDTA. The samples were subjected to agarose gel electrophoresis to determine which digestion time produced the most partial restriction fragments in the 20 to 30 kb size range. Amounts of Ti plasmid DNA (2.5 µg) were then digested with each enzyme in scaled-up reactions incubated for the best time length as indicated by the previous experiment. Reactions were stopped by addition of one volume of phenol saturated with 3 X SSC and extraction by inverting the tubes for 1 to 2 min. The emulsions were centrifuged in a Fisher microcentrifuge for 5 min at RT. The aqueous phase was removed and extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The aqueous phase was removed, and mixed with one tenth volume 3 M sodium acetate and two volumes ice cold ethanol in clean microcentrifuge tubes, followed by incubation at -70°C for at least one hour. The tubes were centrifuged for 10 min at 4°C in a microcentrifuge and the DNA pellets were air dried. The DNA samples were redissolved on ice using 12.5 µl distilled water. pVK102 vector DNA (7.5 µg) was digested to completion with HindIII, while similar amounts of pCP13 were separately digested with BamHI and EcoRI. The three reactions were
stopped by addition of an equal volume of phenol and the aqueous phases extracted as described above. The DNA was precipitated with ethanol. The vector DNAs were redissolved on ice in 32 µl of distilled water, and a 10 x stock solution of calf intestinal alkaline phosphatase (CIP) buffer (4 µl, 0.5 M Tris-HCl, 10 mM MgCl₂, 1 mM ZnCl₂, 10 mM spermidine, pH 9.0, ref. 119) was added. The DNA samples were treated with CIP (20 units, 4 µl, Boehringer Mannheim) in 40 µl reactions, followed by phenol extraction and ethanol precipitation. The phosphatased DNA samples were redissolved on ice in 12.5 µl of distilled water. Vector and insert DNAs cleaved by the same enzyme were mixed and incubated at 65°C for 5 min, followed by 10 min at RT and 20 min on ice. Ligation buffer [10X stock, 3 µl; 66 mM Tris-HCl pH 7.5, 50 mM MgCl₂, 50 mM dithiothreitol, 10 mM adenosine triphosphate (ATP), ref. 119] and T4 DNA ligase (2 units, BRL) were added followed by incubation overnight at 15°C. A portion of the ligation mixture (5 µl) was combined with the two lysogenic E. coli packaging extracts according to the directions supplied by the manufacturer (Amersham). Lambda diluent (500 µl, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 50 mM MgSO₄, 0.01% gelatin) was added. The phage (150 µl) and 500 µl of an overnight culture of strain DH1, grown at 37°C with aeration in LB containing 0.4% maltose, were mixed and incubated at 37°C for 20 min. The mixture of infected cells was added to 3 ml LB and incubated with aeration for approximately one hour. The cells were then spread on selective media (NA plus tetracycline).

Partial digestion of clone pTHH2 to generate subclone pTHH206 was performed by incubating pTHH2 DNA (approx 1 µg) with HindIII (approx. 10 units) in a 60 µl reaction volume at 37°C. At 1, 2, 4, and 8 min, 15 µl
samples were removed and extracted with equal volumes of phenol-
chloroform-isoamyl alcohol [PCI, phenol equilibrated with 50 mM Tris-HCl
pH 8.0 and mixed 1:1 with a chloroform/isoamyl alcohol solution (24:1)]
to stop the reactions. One third of the aqueous phase of each sample was
subjected to agarose gel electrophoresis. The 2, 4, and 8 min samples
showed the largest concentrations of partially digested fragments and were
pooled. The DNA was precipitated from ethanol for 15 min on powdered dry
ice followed by centrifugation for 10 min in a microcentrifuge. The DNA
pellet was washed with ice cold 70% ethanol (100 µl) and dried. After
redissolving the pellet in 20 µl LTE, a portion of this partially digested
DNA (approx. 0.2 µg) was ligated with an equal amount of HindIII-digested
pSa4ΔH vector DNA in a 90 µl reaction at 12°C overnight, followed by
transformation into strain HB101, with Cm as the selection. Subclonings
into vectors pBR322 (15), pVK101 (108), pCP13, pCP13/B (43), pSa152 (173)
and pSa4ΔH were performed using standard techniques (119). Ligations were
performed overnight at 4°C in 10 µl reaction volumes containing Ligation
Buffer, 1 to 2 units of T4 DNA ligase, ATP (1 mM, added from a freshly-
made 10 X stock), approximately 0.1 µg restricted vector DNA and about a
five-fold excess of plasmid DNA containing the fragment of interest. When
the inserted segment disrupted an antibiotic resistance gene, colonies
from transformations with the ligation mixture (5 µl) were first patched
to NA plates containing that antibiotic to determine which clones
contained inserts. Only those isolates containing inserts, as indicated
by an antibiotic-sensitive phenotype, were further analyzed. Culture
tubes containing LB (1.5 ml) and appropriate antibiotics were each
inoculated with cells from groups of 5 transformant colonies. After
growth, plasmid DNA was purified from the cultures, digested with restriction endonucleases, and subjected to gel electrophoresis. Bacteriophage λ DNA (BRL), cleaved with HindIII, was coelectrophoresed with the plasmid DNAs. Colonies from pools that contained the desired subcloned fragment were then individually analyzed to find the clone of interest.

Restriction enzyme cleavage sites in DNA fragments were mapped by double digestions using two methods. First, restriction fragment subclones were digested with two different restriction enzymes. Digestion was simultaneous if the salt and temperature conditions were the same. If the conditions were different, digestion with the enzyme requiring the lower salt was performed first. Appropriate salts were then added (usually 1/20th volume of a 20X stock solution) to bring the salt conditions to the correct level for the second enzyme, which was then added.

The second technique involved excision of electrophoretically separated digestion products of one enzyme from agarose gels, followed by extraction (10) and digestion with a second enzyme. Cosmid clone DNA (approx. 1 to 2 µg) were digested with one restriction enzyme, separated by agarose gel electrophoresis, and stained with ethidium bromide. Fragments visualized with ultra-violet light were cut from the gel with a razor blade and placed in 1.5 ml microcentrifuge tubes. The tubes were frozen in a dry ice-ethanol bath for 30 min, then thawed at RT. The agarose in each tube was thoroughly mashed with a round-ended glass rod. Phenol/chloroform/isoamyl alcohol (PCI, 100 µl) was added and the tubes were vortexed 10 seconds. Following a 15 min incubation in dry ice-
ethanol, the tubes were centrifuged at RT for 15 min in a Fisher
microcentrifuge. The aqueous phase was transferred to a clean tube and
phenol extracted twice more. The DNA was then precipitated from the
aqueous phase with ethanol, washed with 70% ethanol, dried, and
redissolved in 10 µl LTE. These samples were then digested by the second
restriction enzyme and the products separated by electrophoresis.

2.7. Plasmid transfer.

Transformation of *A. tumefaciens* strains with plasmid DNA was
performed as described by Holsters et al. (82). Transformation of *E. coli*
was performed as described by Davis et al. (39). Plasmids were mobilized
from *E. coli* strain DH1 to *Agrobacterium* using the pRK2013-based
triparental mating procedure of Ditta et al. (45). To perform triparental
matings, overnight cultures (2 ml LB plus appropriate antibiotics) of *E.
coli* strain 1231(pRK2013), the *E. coli* strain (usually HB101) containing
the pRK290 derivative plasmid (36, 108) to be mobilised, and the
*Agrobacterium* recipient strain (NT1, UIA143, or K439), were grown to mid-
log phase (determined visually) in LB without antibiotics. Cells were
harvested by centrifugation at 1000 X g for 10 min at RT. The pellets
were resuspended in an equal volume of LB, and the centrifugation and
resuspension were repeated. Samples (3 µl) of each culture were placed
together on a NA plate and incubated at 28°C overnight. The cells were
collected with a sterile cotton swab (Scientific Products Division, Baxter
Healthcare Corp., McGaw Park, IL) and resuspended in 1 ml sterile 0.9%
NaCl. Samples (100 µl of appropriate dilutions) were spread on AB plates
containing selective antibiotics (tetracycline at 2 µl of appropriate
dilutions) were spread on AB plates containing selective ag/ml, others
when appropriate) and incubated at 28°C. Colonies were streaked for isolation on the same medium and used to inoculate 1.5 ml LB cultures for plasmid isolation. Plasmid DNAs purified from the cultures were analyzed by restriction enzyme digestion and agarose gel electrophoresis as described above.

Ti plasmid matings were performed using the drop method of Ellis et al. (47). Instead of resuspending cells of the donor strain from solid media to use in matings, donor and recipient strains were grown separately to midlog phase in ATN plus 0.2% mannitol and the recipient (100 µl) spread over an entire plate of ATN selection medium containing nopaline (2 mg/ml), rifampicin and streptomycin. Following one day of incubation at 28°C, the donors (10 µl) were placed in discreet spots on the recipient lawn. Just prior to placing the donor cells on the recipient lawns, samples were removed, diluted in 0.9% NaCl, spread on NA plates, and incubated at 28°C to determine the number of viable input donor cells. Transconjugant colonies using nopaline as sole carbon and energy source (Noc•) were counted using an Olympus SZH dissecting microscope (Olympus Optical Co., Ltd., Japan) to determine the number of transconjugants per input donor.

2.8. Transposon mutagenesis.

Mutagenesis of pTHB112 with Tn3-HoHol, which encoded resistance to ampicillin and carbenicillin, was performed as described by Stachel et al. (165). Donor strains 1231(pRK2013) and HB101(pHoHol, pSShe, pTHB112) and recipient strain C2110 were grown to midlog phase in LB (20 ml) containing appropriate antibiotics. Filter matings were performed using a glass filter apparatus and 0.45 µm pore 2.5 cm diameter nitrocellulose filters
(Millipore Corp., Bedford, MA). Filters were incubated 4 h at 28°C on an NA plate, and the cells resuspended from them in 1 ml 0.9% NaCl. Cells were spread on NA plates containing ampicillin, tetracycline and nalidixic acid. Transconjugants containing transposon insertion plasmids were verified by digestion of partially purified plasmid DNA from small (1.5 ml) cultures with restriction enzymes BamHI, EcoRI, HindIII and SmaI, followed by agarose gel electrophoresis. Restriction patterns of insertion plasmids were compared to those of the unmutagenized clone pTHB112. To map the insertion positions and orientations of the transposons, individual clone DNAs were first digested with BamHI and the products separated by electrophoresis, as Tn3-HoHol has one asymmetric BamHI site. One of two possible insertion positions was eliminated by separate digestion with the other three enzymes.

Transposon Tn5, which confers resistance to both kanamycin and neomycin, was used to mutagenize pTHH206, essentially as previously described (12, 58, see above). pTHH206 confers resistance to both chloramphenicol and kanamycin, but not to neomycin. Overnight matings on NA plates were performed on filters between E. coli strain 1839(pJB4JI) and A. tumefaciens strain C58C1RS(pTHH206). Cells from filter matings between these two strains were spread on NA plates containing neomycin, chloramphenicol, rifampicin and streptomycin and incubated for 3 to 4 days at 28°C. Transconjugants washed from the plates were inoculated into 200 ml LB containing the four antibiotics and grown overnight at 28°C. In order to isolate Tn5 insertions into the target plasmid, plasmid DNA isolated from the harvested cells and purified through one cesium chloride-ethidium bromide gradient was used to transform two hosts. One
host was *A. tumefaciens* strain NTL, employing neomycin chloramphenicol selection, and the second was *E. coli* strain HB101, using kanamycin or neomycin selection at 20 µg/ml on LA.

2.9. **Determination of β-galactosidase activity.**

β-galactosidase activity in the insertion mutants was qualitatively assayed on Stonier's medium indicator plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, 40 µg/ml). X-gal (1 mg) was dissolved in 100 µl dimethyl formamide and spread on a Stonier's medium plate. β-galactosidase activity was quantitated in lysed cell suspensions essentially as described by Miller (128). *Agrobacterium* strains were grown in Stonier's medium (4 ml) and *E. coli* strains were grown in EC medium (10 ml), containing appropriate antibiotics, to an O.D.₆₀₀ of 0.4 to 0.6 in 125 ml culture flasks or 250 ml nephelometric flasks (Bellco). The O.D.₆₀₀ for the 125 ml flask cultures was measured by aseptically removing a sample (4 ml) to a sterile 100 X 7.5 mm glass tube, measuring the optical density, and returning the culture to the flask. Culture samples (0.2 ml) were added to 1.8 ml Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercapto-ethanol, pH 7.0, ref. 128) in 100 X 7.5 mm glass tubes, followed by addition of 4 drops of chloroform and 2 drops of 0.1% SDS. The tubes were vortexed for 10 seconds and incubated in a water bath at 28°C for 5 min. Ortho-nitrophenyl β-D-galactopyranoside (ONPG, 0.4 ml of a 4 mg/ml solution in 0.1M potassium phosphate, pH 7.0) was added and the time was recorded. The tubes were gently mixed and returned to the water bath. After a yellow color had appeared, the time was again recorded, 1 ml of 1M NaCO₃ was added and the tubes gently vortexed. The A₄₂₀ and A₅₅₀ of each sample were determined (Spectronic 20D
or model 2400 Beckman DU spectrophotometer) and used along with the difference between the two times recorded to calculate values proportional to the β-galactosidase units per cell per minute (128).

2.10. Homogenotization.

Homogenotization of Tn3-HoHol insertions in pTHB112 into pTiC58 was accomplished using the technique described by Ruvkun and Ausubel (146). The transposon insertion plasmid pTHB476 was transformed into strain C58, using carbenicillin as the antibiotic selection. The IncP1 plasmid pPH1JI, which is incompatible with pTHB476, was introduced into strain C58(pTHB476) in a drop method mating, selecting for maintenance of pPH1JI, and for the presence of Tn3-HoHol, on MH medium plates containing gentamicin and carbenicillin. This selects for transconjugants that have undergone a homologous single or double recombinational event between pTHB476 and pTiC58. Transconjugants were analyzed by patching onto AB plates containing tetracycline, and for β-galactosidase activity by patching onto X-gal indicator plates. The isolates were tested for sensitivity to agrocin 84 in the plate bioassay described in section 2.13.

2.11. High voltage paper electrophoresis (HVPE).

Electrophoresis on Whatman No. 1 or 3MM papers (Whatman International Ltd., Maidstone, England) was carried out at 1000 V or 4000 V in Savant TLE 20 and LT-48 electrophoresis chambers, respectively, each containing EC-123 coolant (Savant). Buffer systems employed were: 0.76 M formic acid/1.03 M acetic acid pH 1.7; 1.1 M acetic acid, 0.7 M formic acid pH 1.8 (used in the LT-48); 0.1 M ammonium bicarbonate adjusted to pH 9.2 with concentrated ammonium hydroxide; and 0.2 M boric acid/0.2 M ammonium hydroxide, pH 9.2 (Y. Dessaux, personal communication, refs. 50,
Samples were spotted 1 cm apart along a pencil line drawn across the middle of a paper 28 cm in length and of varying widths, depending on the number of samples. Samples (2 µl) of a reference marker stock solution (5 mg/ml Orange G, 5 mg/ml Xylene cyanole, 2 mg/ml fructose and 2 mg/ml deoxyadenosine) were applied to outer lanes of the paper prior to electrophoresis (50, 148). Once the spots were dry, the paper was wetted by capillary action in electrophoresis buffer. Excess buffer was removed by blotting the papers between paper towels. Papers were secured in the chamber and electrophoresed until the Orange G marker had migrated approximately 9 cm in the TLE 20 chambers, and 12 cm in the larger LT-48 chamber.

To stain electrophoretograms with alkaline silver nitrate, which detects reducing sugars (185), the air-dried paper was dipped in a solution made by dissolving 4 g silver nitrate in 40 ml distilled water and mixing it with 2 L acetone. Papers electrophoresed in ammonium borate buffer were dipped for approximately 2 min in a 40 mM H₂SO₄, 95% ethanol solution to remove the borate which interferes with silver nitrate staining (Y. Dessaux, personal communication), and thoroughly dried prior to staining with silver nitrate. After air drying, the paper was dipped in a 90% ethanol, 2% NaOH solution and then steamed over a beaker of boiling water while still wet. The stained paper was dipped in fixer (Eastman-Kodak), rinsed thoroughly in tap water, and dried. Sugars were detected by dipping electrophoretograms in a solution made by mixing 5 g p-anisidine-HCl, 5 ml concentrated HCl, and 220 ml ethanol. The paper was dried and heated at approximately 110°C for 3 to 5 min (85). Amino acids were detected by dipping papers in ninhydrin reagent (0.25%
ninhydrin in acetone) followed by heating at 110°C for 1 to 3 min (174). phosphorylated compounds were visualized by dipping papers in a solution made by mixing 40 ml phosphomolybdate reagent and 160 ml acetone, heating the dried papers at 60°C for 10 to 15 min, and irradiating them for several min with ultraviolet light (TM-36 transilluminator, Ultra-Violet products, Inc.). Phosphomolybdate reagent was prepared by dissolving 50 g Na$_2$MoO$_4$.2H$_2$O in 250 ml distilled water. To this was added 500 ml 1 N HCl and 210 ml 72% HClO$_4$. The solution was diluted 1:2 with distilled water prior to use (70).

2.12. **Descending Paper Chromatography**

Chromatography on Whatman No. 3 paper was carried out overnight in a Model 305 chromatography chamber (Research Specialties Co., Dayton, Ohio) using a solvent composed of 3 volumes 1-butanol, 1 volume glacial acetic acid and one volume distilled water. The paper was 45 cm long and samples were spotted 2 cm apart along a pencil line drawn 8 cm from one end. Dye markers (2 µl, see above) were spotted on either side. Triangular notches 1 cm wide were cut out along the end of the paper nearest the samples to promote straight sample migration (Y. Dessaux, personal communication). A tray of solvent (200 to 300 ml) was placed in the bottom of the chamber, and the chamber sealed for approximately one hour at RT to equilibrate. The notched end of the paper was immersed in the solvent in the upper solvent container of the chamber and held there with a glass rod. The remainder of the paper was draped over a second fixed glass rod and suspended over the tray of solvent in the bottom of the chamber. The chamber was sealed and chromatography allowed to proceed overnight at RT until the xylene cyanole dye marker had traversed
approximately two thirds of the paper. Staining was performed as with paper electrophoretograms.

2.13. Agrocin 84 assays.

Sensitivity to agrocin 84 was determined as follows. A colony of the producer strain, NT1(pAgK84-A1), was suspended in 0.5 ml 0.9% NaCl, 4 µl were inoculated onto the center of Stonier's medium plates and the plates incubated at 28°C for 48 h. Cultures (LB, 2.0 ml) of indicator strains were prepared and incubated 24 to 48 h with aeration at 28°C. For the assay, the Stonier’s plates were exposed to chloroform vapors to kill the producer strain by inverting them over chloroform-soaked squares of paper towel for 10 min, followed by airing for 5 min. The overnight cultures of indicator strains were centrifuged at 1000 X g for 10 min, and the cell pellets resuspended in 2.0 ml 20 mM potassium phosphate buffer, pH 7.0. Tubes of molten soft agar [0.7% (w/v), in 20 mM potassium phosphate buffer pH 7.0] kept at 48°C were inoculated with 100 µl of the indicator cell suspensions and overlayed onto the prepared Stonier’s plates. When required, strips of Whatman No. 1 paper 3 cm X 0.3 cm soaked with 10 nmoles of a partially purified mixture of agrocinopines A+B were dried and sterilized in a glass petri dish containing a chloroform-soaked square of paper towel for 10 min. After airing for 5 min, the strips were aseptically placed radially on the plates, which were then incubated at 28°C for 2 to 3 days. Induction of sensitivity by the opine was indicated by increased inhibition zones adjacent to the paper strips as first described by Ellis and Murphy (50).
2.14. **Agrocin 84 secretion assay.**

To determine the ability of strains harboring a pAgK84 derivative and a second plasmid to secrete agrocin 84, cell suspensions of these strains were inoculated onto Stonier's plates and incubated at 28°C for two days. The cells were then killed and the plates overlaid as in the agrocin 84 sensitivity assay, using strain K439 as the sensitive indicator.

2.15. **Agrocin 84 uptake.**

Uptake of agrocin 84 was inferred by measuring disappearance of the toxic agent from culture supernatants following incubation with a high density of cells. Strains to be tested were grown in Stonier's medium at 28°C to an OD₆₀₀ of approximately 0.4. Culture samples (2 ml) were centrifuged for 10 min at 1000 x g and resuspended in 40 µl Stonier's medium. Cell suspensions (27 µl) were added to 3 µl chloroform-sterilized partially purified agrocin 84, mixed by pipetting and incubated at 28°C. Samples (10 µl) were taken at various times, centrifuged for 2 min in a Fisher microcentrifuge and the supernatants spotted on Stonier's medium plates. After allowing 5 to 10 min for the samples to be completely absorbed into the agar, the plates were chloroform-sterilized and overlaid with strain K439 as the indicator.

2.16. **Agrocinopine uptake.**

Cells were grown in Stonier's medium at 28°C to early exponential phase (approximately 2 X 10⁸ cells/ml). The cells were harvested by centrifugation, and washed once with and resuspended in the same volume of AT medium without a carbon source. The cells were inoculated (1 µl) into 30 µl ATN medium containing 3 mM agrocinopines A+B in a sterile 1.5 ml plastic microcentrifuge tube. The cultures were incubated at 28°C on
a shaker at 150 rpm, and 10 µl samples were removed at 0, 18 or 24, and 48 h and analyzed by HVPE for disappearance of the agrocinopines.

2.17. **Agrocinopine catabolism.**

To determine ability of *Agrobacterium* to grow using agrocinopine B as a sole carbon and energy source, strains were grown at 28°C in ATN medium containing 0.2% glucose to midlog phase, harvested by centrifugation, and washed twice with AT salts. Microcentrifuge tubes containing 15 µl of ATN medium plus 5 mM agrocinopine B, purified as described above, were inoculated with 1 µl of washed cells and incubated at 28°C. Samples (2 µl) were removed every 24 h with calibrated glass capillary pipets and appropriate dilutions, using 0.9% NaCl, were spread on NA plates. The plates were incubated at 28°C to determine the number of viable cells per ml. Additional 2 µl samples were removed at selected timepoints and subjected to HVPE to ascertain opine uptake.

2.18. **Digestion of agrocinopine B by cell-free extracts.**

Catabolism of agrocinopine B *in vitro* was examined by growing strains K439 and NT1 in 250 ml Stonier's medium to mid log phase. The cells were harvested by centrifugation at 6000 X g for 10 min at 4°C. The cell pellets were resuspended in 100 ml 0.9% NaCl and recentrifuged. The washing procedure was repeated. After decanting the supernatant and removing any remnants with a pasteur pipette, the cell pellet was resuspended in 1.5 ml ice cold Extraction Buffer (EB; 100 mM Tris-HCl pH 7.5, 2 mM dithiothreitol, 1% ethylene glycol, 0.2 mM phenylmethyl-sulfonyl fluoride) and transferred to 30 ml Corex centrifuge tubes on ice. The cells were disrupted on ice by sonication using a Branson model 450 sonifier (Branson Ultrasonics, Danbury, CT) with a tapered micro tip.
Sonication was performed on the lowest output setting for three 30 second intervals separated by 30 second cooling intervals, with care being taken to avoid foaming. The tubes were then centrifuged at 4°C for 15 min at 6700 X g to remove undisrupted cells and particulate debris. As an alternative to sonication, cells were made permeable with toluene. To cell pellets resuspended in 1 ml cold EB was added 100 µl toluene, followed by vigorous vortexing for 30 seconds to form an emulsion.

Samples of the supernatant or emulsion (95 µl) were mixed with 5 µl agrocinopine B purified as described above. The agrocinopine/cell extract mixtures were incubated at 28°C and the reactions stopped by addition of 20 µl 2 M sodium acetate. The stopped reactions were incubated on ice for 10 min to precipitate proteins followed by centrifugation in a microcentrifuge for 5 min. To visualize the opine substrate and potential metabolic intermediates, reaction supernatants were spotted on papers and electrophoresed in ammonium borate or formate/acetate buffers, or subjected to descending paper chromatography using a butanol/acetate solvent, as described in sections 2.11 and 2.12.

2.19. Analysis of proteins.

Three strategies were used to visualize proteins encoded by recombinant clones. The first entailed isolating cloned EcoRI, BamHI, and HindIII restriction fragments under the control of an inducible promoter, the tac promoter of plasmids pMMB22 and pMMB24 (6). Plasmid-containing strains were grown to mid-log phase in LB cultures (2 ml) with or without isopropyl-β-D-thio-galactopyranoside (IPTG, 5 mM) at 37°C. Samples (100 µl) were removed and the cells harvested by centrifugation for 2 min in a microcentrifuge at RT. The cell pellets were resuspended
in 50 µl Laemmli sample buffer (LSB, 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% BPB, ref. 111), and boiled for five min. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (111), using slab gels. The running gels (12.4 cm X 14 cm, 12.5% acrylamide) were 1.5 mm thick. The stacking gel (3% acrylamide) was 1 cm in height. Electrophoresis of 20 µl samples was performed for 5 h using 20 mA constant current, in a Hoeffer SE 600 electrophoresis chamber containing running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS pH 8.3). SDS was included only in the upper buffer chamber. Following electrophoresis, the stacking gel was removed using a razor blade, and the gel was stained overnight using Solution A [0.25% (w/v) Coomassie Brilliant Blue, 45% (v/v) methanol, 0.9% (v/v) acetic acid, ref. 154]. Destaining was performed in Solution B [45% (v/v) methanol, 0.9% (v/v) acetic acid] for approximately two days on a slowly rotating platform at RT.

Plasmid-encoded proteins were also visualized using the maxicell technique (149) as described by Silhavy et al. (158). Cultures of strain LCD44 containing different plasmids were grown to an O.D.₆₀₀ of 0.6 to 0.7 in 250 ml nephelometric flasks containing M63 medium (10 ml) supplemented with 0.4% glucose, 20 µg/ml methionine, and appropriate antibiotics but lacking casamino acids. Immediately following growth, cells were irradiated with ultraviolet light in open petri plates on a rotary platform at approximately 80 rpm. The ultraviolet light source consisted of a bank of 6 General Electric germicidal lamps emitting 86% of their radiant power at 253.7 nm (186). The fluence rate was measured at 1 J/second/m² using a DRC-100X Digital radiometer equipped with a DIX-254
sensor (Spectroline™, Spectronics Corp., Westbury, NY). The cells were exposed to ultraviolet light for approximately 2.5 min and transferred to foil-covered tubes. After a 30 min incubation at 37°C with aeration, cycloserine (40 µl of a freshly-made 50 mg/ml stock in distilled water) was added and incubation continued overnight. Samples of each culture (100 µl) were spread on NA plates and incubated at 37°C to determine viability. The remainder of each culture was centrifuged at RT for 10 min at 1200 X g. To starve the cells for methionine, the cell pellets resuspended in 2 ml M63 medium lacking this amino acid and incubated on a shaker at 37°C for 60 min. 35S-methionine (Tran35S-label™, 1036 Ci/mmol, ICN Biochemicals, Inc., Irvine, CA) was added to a concentration of 25 µCi/ml and incubation was continued for 30 min. The cells were harvested by centrifugation, washed twice with 0.5 ml M63 salts, and transferred to 1.5 ml microcentrifuge tubes. The cell pellets were resuspended in LSB, boiled for 5 min, and used immediately or stored at -20°C.

Proteins encoded by recombinant plasmids were visualized by a third technique, transcription and translation of the plasmids in vitro using a commercial transcription and translation kit (Amersham). The in vitro transcription and translation assay was performed exactly as described by the manufacturer. Samples were boiled in LSB as described above. The amount of radioactivity in samples of boiled protein samples from each maxicell or in vitro transcription and translation extract (2 µl) was determined using a Bioscan QC-4000 XER counter (Bioscan, Inc., Washington, D.C.), and an equal amount of radioactivity (approximately 20,000 cpm) for each sample was loaded onto the gel. Electrophoresis was performed as described above. Following electrophoresis, the stacking gels were
removed using a razor blade, and the gels were fixed overnight in 10% acetic acid, 5% methanol (100 ml) on a slowly rotating platform. The gels were soaked for 30 min in DMSO (100 ml) on the rotating platform. This was repeated with fresh DMSO. The gels were then immersed in DMSO containing 20% PPO (100 mls) for 3 h on the platform. The DMSO/PPO was replaced with distilled water and the gels were washed with 4 changes of water for one hour (17). The gels were then mounted on rectangles of Whatman 3 MM paper cut approximately 4 cm larger in length and width than the gels. Drying was performed over a 2 h period, using a Welch Duo-Seal vacuum pump, connected through a Savant refrigerated condensation trap to a Hoeffer SE 1160 slab gel drier set at 50°C. The dried gels were exposed to XAR 5 film (Eastman-Kodak) between two intensifying screens (Cronex™, Du Pont) at -80°C for 2 to 8 days.

2.20. Southern hybridizations.

Southern hybridizations (164) were performed according to the instructions of the manufacturer of the membrane used [Genescreen™ Instruction Manual, New England Nuclear™ (Du Pont)]. Equivalent amounts of plasmid DNA were digested with appropriate restriction enzymes, and fragments separated by electrophoresis on 0.7% agarose gels as described above. Following staining with ethidium bromide and photography, the gels were denatured by incubation with gentle agitation at RT in 0.2 N NaOH, 0.6 M NaCl for 30 min, followed by a one hour wash in 0.025 M Na₂HPO₄/NaH₂PO₄ (pH 6.5) with a change of buffer every 15 min. Transfer to Genescreen™ membranes was performed overnight using 1X SSC. Probe DNA was radioactively labeled by nick translation according to the instructions of the manufacturer [nick translation kit from BRL, ³²P-dCTP
(650 Ci/mmole) from ICN). Unincorporated radioactive nucleotides were separated by passing the DNA through a column (10 ml) of Sephadex G50 resin (Pharmacia Inc., Piscataway, NJ) equilibrated with TE. One half of the eluted labeled DNA was used per hybridization. Prehybridization, hybridization and posthybridization procedures were carried out exactly as described in section II, Method III of the Genescreen™ Instruction Manual. The membranes were wrapped in plastic wrap and exposed to XAR5 film between two intensifying screens at -80°C. Autoradiograms were developed in GBX developer (Eastman-Kodak) for 3 to 5 min. The autoradiograms were transferred to a 10% acetic acid bath for one minute, then to a fixer bath (Eastman-Kodak) for 4 to 5 min. The film was rinsed under running tap water for several minutes before drying.
CHAPTER III

RESULTS

3.1. Isolation of cosmid clones conferring agrocin 84 sensitivity.

The eight o'clock region of the pTiC58 map (Fig. 2) has been associated with three characteristics: agrocinopine catabolism (50), agrocin 84 sensitivity (83), and induction of Ti plasmid conjugal transfer (47, 50, 51). It has been proposed that one transport system mediates the uptake of both agrocin 84 and agrocinopine A (50). In order to determine the genetic relationship between agrocin 84 sensitivity and uptake, and agrocinopine catabolism, we set out with the goal of cloning the region encoding these three phenotypes. To clone the region, partial BamHI, EcoRI and HindIII digests of pTiC58 were ligated into cosmids pVK102 and pCP13 as described in Materials and Methods. Cosmids packaged using a commercial kit were used to infect E. coli strain DH1, grown on LB supplemented with maltose to induce synthesis of the bacteriophage λ receptor. Infected cells (100 µl) were spread on NA plates containing Tc and incubated at 37°C. Colonies from the plates were used to inoculate small (1.5 ml) LB cultures. Isolated DNAs from cultures of 145 clones were digested with the restriction enzyme used in their construction, and the fragments separated on agarose gels. pTiC58 DNA was digested with the same enzyme and coelectrophoresed with the cosmid clone DNAs. Restriction fragments of pTiC58 that comigrated with cosmid clone fragments were marked on the pTiC58 map of Depicker et al. (41). A contiguous cluster of marked fragments for a particular cosmid clone was taken to indicate
Figure 2. Restriction map of pTiC58 showing overlapping cosmid clones. Clones were constructed from partial restriction endonuclease digestion fragments of pTiC58 DNA using BamHI, EcoRI, and HindIII. Fragments were ligated with linearized cosmid vectors pCP13 and pVK102, and mapped as described in Materials and Methods. Cloned inserts are shown as arcs with the associated plasmid name. Adapted from Depicker et al. (41) and Nester and Kosuge (135), modified according to Beck Von Bodman et al. (8) and this work. Acs, agrocinopine synthesis; Ape, bacteriophage API exclusion; Agr\textsuperscript{i}, agrocin 84 sensitivity; Inc, incompatibility; Noc, nopaline catabolism; Nos, nopaline synthesis; Tra, conjugal transfer; Vir, virulence.
pTiC58

- BamHI
- EcoRI
- HindIII

T DNA

- pTHE16
- pTHE7
- pTHE17
- pTHH26
- pTHH23
- pTHH6
- pTHH21
- pTHH32
- pTHB102
- pTHB112
- pTHB55
- pTHE12
- pTHB94
- pTHH11
the insert location for that clone. This was confirmed by digestion with additional enzymes. Only five of the clones examined appeared to lack inserts. At least 47 clones contained inserts with fragments mapping to two or more different sections of the Ti plasmid. These were presumed to contain noncontiguous regions of Ti plasmid DNA and were not examined further. The size of inserted DNAs ranged from 17 to 35 kilobases (kb) in length. Recombinant plasmids containing overlapping inserts covering the entire Ti plasmid were identified (Fig. 2).

Determinants for the agrocinopine-agrocin 84 region, acc, have previously been positioned by large deletions and two imprecisely-mapped transposon insertions to lie between kb coordinates 127 and 134 of pTiC58 (30, 50, 83). Several clones containing inserts encompassing this region were mobilized into Ti-plasmidless A. tumefaciens strain NT1 and tested for their ability to confer sensitivity to the antibiotic agrocin 84. pTHBl12 and pTHH32 are two recombinant plasmids that conferred agrocin 84 sensitivity (Agr") on strain NT1 (Figs. 3A and 4). These plasmids did not confer sensitivity on E. coli strain DH1, although this strain was sensitive to a product presumably encoded by the genome of Agrobacterium strain NT1 (Fig. 3B). Plasmids pTHB55 and pTHE16 were among those that did not confer agrocin 84 sensitivity (Agr) on strain NT1 (Fig. 3A). Inhibition zone diameters exhibited by active clones in strain NT1 were intermediate between those of A. tumefaciens strain C58 and the supersensitive strain K439, but agrocin 84 sensitivity conferred by these clones remained inducible by agrocinopines A+B as determined by the plate bioassay described in Materials and Methods section 2.13 (Fig. 3A). The relative map positions of the eight cosmid clones used in this work and
Figure 3. A. Agrocin 84 bioassays of Agrobacterium strain NT1 containing recombinant clones. Assays were performed as described in section 2.13. Strains tested in the overlays were: 1, NT1; 2, C58; 3, K439; 4, NT1(pTHB55); 5, NT1(pTHB112); 6, NT1(pTHE16); 7, NT1(pTHH32). B. Agrocin 84 bioassay of E. coli strain DH1 containing an acc clone. EC plates plus thiamine (10 µg/ml) were inoculated centrally with NT1 (plates 1 and 3) or NT1(pAgK84-A1) (plates 2 and 4), and incubated at 28°C. Overlays were prepared identically to Agrobacterium overlays except that the overnight cultures were grown at 37°C. Plates were incubated at 28°C. Strains used to seed overlays were: 1 and 2, DH1; 3 and 4, DH1(pTHB112). All paper strips were impregnated with 10 nmoles of agrocinopines A+B.
Figure 4. Map positions of recombinant clones defining the pTiC58 agrocinopine-agrocin 84 region and the agrocin 84 phenotypes they confer. The plasmids were tested in strain NT1 as described in Materials and Methods. Agr'; agrocin 84 resistant, Agr'; agrocin 84 sensitive. The shaded box at the top indicates the location and minimum size of the agrocin 84 sensitivity locus. The maximum size of the region is shown by the flanking open boxes. Coordinates (in kb) correspond to the map of pTiC58 (41).
the agrocin 84 phenotypes associated with them are shown in Fig. 4. Based on the agrocin 84 sensitivity phenotypes they confer, plasmids pTHB112 and pTHE16 roughly define the left border of the acc region as lying between the left ends of BamHI fragment 14 and EcoRI fragment 16. pTHB55 and pTHH32 confine the right border to a region between the right ends of BamHI fragment 5 and HindIII fragment 11. The maximum size of the region as defined by these clones is 13.5 kb, indicated by the open box in Fig. 4.

To test the stability of one of these plasmids in strain NT1, a strain containing cosmid clone pTHH2 was grown overnight at 28°C in LB (2 ml) without antibiotics. This culture was used to inoculate a second overnight culture (1:100 dilution). Following overnight growth, a sample of the second culture was spread on an NA plate, and 50 isolated colonies were patched with toothpicks to plates containing NA and NA plus tetracycline at 2 µg/ml. Of the 50 colonies tested, 11 were sensitive to tetracycline, indicating that they had lost the plasmid.

In summary, cosmid clones of pTiC58 containing inserted DNA mapping approximately to the 122.5 to 136 kb coordinates of the Ti plasmid confer sensitivity to agrocin 84 on Agrobacterium strain NT1. Since this sensitivity remains inducible by agrocinopines, the regulatory apparatus for acc appears to be encoded by these plasmids. The region does not confer agrocin 84 sensitivity on E. coli. These plasmids seem to be somewhat unstable in Agrobacterium, as approximately 20% of the cells appear to have lost the plasmid after two consecutive overnight culturings, about 30 generations, in nonselective medium. Antibiotic
selection should therefore be employed to maintain the plasmid in all cells of a culture.

3.2. Agrocin 84 secretion

Ellis et al. (51) demonstrated that an Agrobacterium strain containing both the plasmid responsible for agrocin production, pAgK84, and pTiC58Tra⁵ does not detectably secrete any antibiotic into the solid medium on which they are grown. These authors hypothesized that agrocin 84 is being broken down, or that the antibiotic is being secreted, but is rapidly taken back into the cell by the constitutively expressed acc transport system of pTiC58Tra⁵. To determine whether the cloned acc region also interferes with agrocin 84 secretion, a Tn₅ derivative of pAgK84, pAgK84-E2, in which the transposon insertion does not affect agrocin production or immunity to agrocin 84 (58, 147), was introduced into strain NTl containing one of the acc region cosmid clones, pTHB112, pTHH32, pTHB55 or pTHE16. The four strains were tested as producers of agrocin 84 in the standard bioassay. Strain NTl containing pAgK84::Tn₅ and either of the two clones examined that conferred agrocin 84 sensitivity on strain NTl produced less extracellular agrocin 84 than did strain NTl containing only pAgK84::Tn₅ (Fig. 5, B, E, and F). The two clones not conferring sensitivity to agrocin 84 had no detectable effect on the amount of agrocin 84 secreted (Fig. 5, B, C, and D).

A positive correlation therefore exists between the ability of the plasmids tested to confer sensitivity to agrocin 84 and their apparent ability to reduce agrocin 84 secretion by agrocinogenic agrobacteria.
Figure 5. Agrocin secretion reduction by cosmid clones of the ace region. Standard agrocin 84 bioassays were performed, except that the following strains were inoculated in the center of the plates instead of strain NT1-A1: A and B, NT1(pAgK84-E2); C, NT1(pAgK84-E2, pTHE16); D, NT1(pAgK84-E2, pTHB55); E, NT1(pAgK84-E2, pTHB112); F, NT1(pAgK84-E2, pTHH32). Agar overlays were seeded with: A, NT1; B through F, K439.
We found that digestion patterns of some of our clones did not match the map of Buscher et al. (41) exactly. Fine structure mapping of the region with enzymes BglII, EcoRI, HindIII, and Sau3AIII revealed two additional HindIII fragments (Fig. 6A) and confirmed the observation that fragments 3 and 5 did not encode all of acrB and were not analyzed further. The third recombinant plasmid, pTKS206, contained pTCS58 HindIII fragments 3A, 11 and 38 (Fig. 6A) and conferred agrocin 84 sensitivity on strain NT1 (Fig. 6B, plate D). Single fragments generated by digestions with HindIII, EcoRI, or BglII were also cloned into vectors pVK101 and pCP12, pBglII fragment 11 and pBglII fragment 35, inserted into vectors pVK101 and pCP12, respectively, or inserted into pSa152, failed to confer agrocin 84 sensitivity on strain NT1 (Fig. 6B).
3.3. **Revised mapping of the ace region.**

We found that digestion patterns of some of our clones did not match the map of Depicker et al. (41) exactly. Fine structure mapping of the region with enzymes **BamHI**, **EcoRI**, **HindIII**, and **SmaI** was therefore performed by digestion of restriction fragments extracted from agarose gels as described in Materials and Methods section 2.6 (Fig. 6A). This map replaces the one previously published (73). A heretofore undiscovered **HindIII** fragment, 42, was found to lie between **HindIII** fragments 3 and 31a (Fig. 6A this work, ref. 8).

3.4. **Subcloning of the ace region.**

To ascertain the smallest DNA region required for expression of agrocin 84 sensitivity, the products of a partial **HindIII** digestion of cosmid clone pTHH2 were subcloned into pSa4ΔH, as described in Materials and Methods section 2.6. To determine which fragments were cloned in the 20 recombinants analyzed, partially purified plasmid DNAs were digested with **HindIII**, **BamHI**, **EcoRI** and **SmaI**, and fragments separated by agarose gel electrophoresis. Three inserted fragments were isolated in pSa4ΔH. The first two, **HindIII** fragments 3 and 11, carried by plasmids pTHH204 and pTHH205, respectively, did not encode all of **ace** and were not analyzed further. The third recombinant plasmid, pTHH206, contained pTiC58 **HindIII** fragments 31a, 11 and 38 (Fig. 6A) and conferred agrocinopine-inducible agrocin 84 sensitivity on strain NT1 (Fig. 6B, plate 1). Single fragments generated by digestions with **HindIII**, **BamHI**, or **EcoRI** were also cloned into vectors pVK101 and pCP13/B. **HindIII** fragment 11 and **BamHI** fragment 5, inserted into vectors pVK101 and pCP13/B respectively, or inserted into pSal52, failed to confer agrocin 84 sensitivity on strain NT1 (Fig. 6A).
Figure 6. A. Revised restriction map of \textit{acc} region showing subclones and associated agrocin 84 phenotypes in strain NTl. Plasmid names are given to the left of lines indicating restriction fragments they carry. Agrocin 84 phenotypes of strain NTl carrying each plasmid, as determined by the plate bioassay, are given to the right. R, agrocin 84 resistance; S, agrocinopine-inducible agrocin 84 sensitivity. The box at the top indicates the minimum (shaded) and maximum (open) sizes of \textit{acc}. B. Agrocin 84 plate bioassays overlaid with different strains. 1, NTl(pTHH206); 2, K439; 3, C58; 4, NTl(pTHCB17A); 5, NTl(pTHCB17B).
A

Agrocin

pTHCB17A, pTHCB17B
pTHSE28
pTHSE16, pTHVE16
pTHHSB5
pTHSH11, pTHVH11
pTHSS9
pTHH206

BamHI

R

HindIII

5

EcoRI

S

SmaI

R

kb

1

2

3

4

5

14

5

17

7

3

42

31a

11

38

38

17

34

39

33

21

26

16

33

31

23

27

19

9

22

1b

130

140
The same held true for EcoRI fragment 16, cloned into pVK101 or pSal52, and EcoRI fragment 26 and SmaI 9, both cloned into pSal52 (Fig. 6A). Agrocinopines had no effect on these resistant phenotypes.

Sormann (163) reported that BamHI fragment 17, when cloned into pBR322 and the resulting plasmid recombined into a derivative of pSa, confers agrocin 84 sensitivity on a Ti-plasmidless A. tumefaciens strain. We considered this unlikely, as our cosmid pTHE16, which contains BamHI fragment 17, does not confer agrocin 84 sensitivity (Figs. 3 and 4). However, it is possible that a transport function encoded on BamHI 17 cloned in pBR322 could be expressed under the control of a promoter located on the vector; perhaps the promoter for the pBR322 tetracycline resistance gene. To test this, we constructed plasmids functionally equivalent to those of Sorman, containing BamHI fragment 17 in either orientation with respect to the pBR322 tetracycline promoter. Recombinant plasmids pTHCB17A and pTHCB17B contain BamHI fragment 17 inserted into the BamHI site of pBR322 in opposite orientations with respect to the nearby tetracycline gene promoter. These two constructs were each linearized with PstI, which cuts only in pBR322, and inserted into the PstI site of pCP13. This cloning was required in order to maintain the pBR322 replicon in Agrobacterium. In contradiction to the data of Sorman, neither of the two resulting plasmids conferred agrocin 84 sensitivity on strain NT1, either in the absence or presence of antibiotic selection pressure (Fig. 6B, plates 2 through 5).

Comparison of the overlapping cosmid clones along with analysis of pTHH206 indicate that the smallest region that should confer agrocin 84
sensitivity consists of HindIII fragments 31a and 11. This region is indicated by the shaded box in Fig. 6A.

3.5. Agrocinopine uptake by strain NT1 containing recombinant clones.

It has been proposed that the agrocinopine permease is responsible for agrocin 84 uptake (50, 51). If this is true, clones encoding agrocin 84 sensitivity should also encode transport of the opine. Strains containing cosmid clones pTHB55, pTHB112, pTHE16, pTHH32 (Fig. 4) and the subclone pTHH206 (Fig. 6A) were therefore analyzed for their ability to transport the sugar opines. The plasmid-containing strains, grown in Stonier's medium and washed with AT salts, were inoculated into small (30 µl) ATN cultures containing agrocinopines A+B and incubated on a shaker at 28°C. Opine transport was measured by removing samples (10 µl) at different times and subjecting them to HVPE to detect any disappearance of the opine from the culture medium. When incubated in the presence of a 3 mM mixture of agrocinopines A+B, NT1(pTHB112), NT1(pTHH32) and NT1(pTHH206), the three strains sensitive to agrocin 84, removed the opines from culture supernatants within 48 h (Fig. 7A). The agrocin 84-resistant strains NT1(pTHB55) and NT1(pTHE16) contain clones that overlap most of the region from the left and from the right, respectively (Fig. 4). Neither showed detectable removal of agrocinopines A+B from the medium under the same conditions. Instead, they behaved like strain NT1, converting agrocinopine A into a compound with an electrophoretic mobility and staining properties indistinguishable from those of agrocinopine B (ref. 50, Fig. 7A this work). Plasmid pTHB112 tested in E. coli strain DH1 did not confer uptake of agrocinopine A (Fig. 7B).
Figure 7. A. HVPE analysis of agrocinopine A and B uptake by strain NT1 containing clones overlapping the agrocinopine-agrocin 84 region. Electrophoresis was performed in formic acid/acetic acid buffer (pH 1.7) at 1000 V for approximately 30 min. Electrophoretograms were stained with alkaline silver nitrate. Reference markers are fructose (F), Xylene cyanole (XC) and Orange G (OG). Acp A and Acp B are agrocinopines A and B, respectively. Ori marks the origin of electrophoresis. + and - indicate the anode and cathode, respectively. The numbers indicate hours that cultures were incubated with the opines. All clones shown have the prefix pTH-. Results for strain NT1(pTHH206) were similar to those of strain NT1(pTHB112).

B. Agrocinopine uptake assay of *E. coli* strain DH1(pTHB112). Labels are as in part A.
Argocinopine A can be converted to agrocinopine B and glucose

AcpB - AcpA -

OG - xc - Ori -

B55 B112 E16 H32 C58 K439 NT1

B +

AcpA -

OG - xc - Ori -

0 24 48 DH1

(pTHB112)
Each and every plasmid tested in strain NT1 that confers agrocin 84 sensitivity also confers agrocinopine transport, indicating that the two phenotypes are not separable. *ace* does not confer agrocin 84 sensitivity or agrocinopine uptake on *E. coli*.

3.6. Agrocinopine B catabolism in vivo.

We have defined a segment of pTiC58 as being responsible for agrocin 84 sensitivity and agrocinopine transport, yet it remained to be demonstrated that this region actually allows catabolism of the opines. To test this, we determined if appropriate clones would allow strain NT1 to grow on one of the sugar phosphate opines as sole carbon and energy source. Agrocinopine A can be converted to agrocinopine B and glucose by several *Agrobacterium* strains, including NT1 (50), which can grow using glucose as a nutritional source. Therefore, agrocinopine B was used as the nutritional source instead of agrocinopine A.

In preliminary experiments, strain NT1 grew with partially purified agrocinopine B as sole carbon and energy source. This suggested that the opine preparation was contaminated with some utilizable carbon source. Additional purification as described in Materials and Methods was therefore performed. In initial attempts to generate agrocinopine B sufficiently pure for catabolism experiments, agrocinopine A was converted to agrocinopine B and glucose by boiling it in 1.5 M acetic acid for 15 min. The agrocinopine B was separated by preparative HVPE and recovered by elution from strips cut out of the electrophoretogram, as described in section 2.3. Biological purification using strain NT1 was then performed, followed by column chromatography. Agrocinopine B was initially eluted from ion exchange columns using a single buffer concentration (0.2 M
pyridine, 0.1 M acetate). In growth experiments using agrocinopine B preparations purified in this manner, the opine was not removed from the culture medium by strain NT1. However, the preparations still supported the growth of the bacteria (Fig. 8).

To eliminate catabolizable contaminants, three changes were made. The original starting material was changed to an agrocinopine B sample (purified from plant tumors by M. Ryder), and the preparative paper electrophoresis step was eliminated. Elution of the opine from the ion exchange column was performed using a pyridine/acetate step gradient. This presumably eluted contaminants at different buffer concentrations than agrocinopine B. To test these purification improvements, and to determine if the opine can serve as a sole source of carbon and energy, strains C58, NT1, and NT1 containing acc clones pTHB112 or pTHH206 were incubated in 15 µl ATN medium cultures at 28°C containing 5 mM purified agrocinopine B. Samples (2 µl) were removed daily from the cultures, serially diluted and spread on NA plates to quantitate the viable cell concentration as described in section 2.17. At the initial and final sampling times, a second sample was subjected to HVPE to determine opine uptake. Strains C58, NT1(pTHB112) and NT1(pTHH206) all removed the opine from the medium while strain NT1 did not (Fig. 8). All four strains started out at the same cell concentrations, but by 66 h, strains C58, NT1(pTHB112) and NT1(pTHH206) increased to cell densities approximately two to three orders of magnitude greater than that of strain NT1. This shows that the acc region confers on cells the ability to grow using agrocinopines as the sole source of nutrition.
Figure 8. A. Utilization of agrocinopine B as sole carbon source. Growth assays were performed as outlined in Materials and Methods. Open symbols, substrate opine purified using original protocol; solid symbols, substrate opine purified using modified protocol (see section 2.3.). ●, C58; □, ■, NT1; ▲, NT1(pTHB112); ◀, NT1(pTHH206). B. Half of the initial and final timepoint samples were subjected to HVPE in formic acid-acetic acid buffer at 5000 V for approximately 15 min and alkaline silver nitrate staining to show opine transport. Labels are described in the legend to Fig. 7. The numbers indicate hours that cultures were incubated with the opines.
In addition to showing that any escher cells with the ability to produce bacteriophages as a normal event, we have decided to determine, in which the steps of these synthetase are blocked and which of the intermediates are produced during metabolism of host can be detected. Outside of cultures of S. typhimurium (230 ml) were inoculated with strains N71 and K439. Under the microscope, the spread and control of the material in section 2.14. Five strains were mixed in equal proportions in a 24-hour paper incubation. The reactions were either stopped immediately or incubated in a reaction with acetic acid and centrifuged to separate buffer to detect RNA and protein with a visible light microscope. Each lane in a gel contains a converted acetylase isolated from bacteria. In some results, complete bands were converted, and no visible bands were detected. NT1 was isolated from the more active variants. This strain had been converted by transferral. The mixture of pTHB-102 (pTHB-200). Since the plasmid pTHB-102 had no visible effect on the strain, K439 had no visible effect on the strain substrate. Since activity of an isolate could not be detected by using a plasmid without a visible effect on the strain's substrate. Since activity of an isolate could not be detected by using a plasmid without a visible effect on the strain's substrate.
3.7. **Agrocinopine catabolism in vitro.**

In addition to showing that *acc* endows cells with the ability to grow using agrocinopines as a nutrient source, we wanted to determine the way in which the sugar phosphate opines are broken down and to identify the intermediates of agrocinopine catabolism. Incubation of agrocinopines with cell-free *Agrobacterium* extracts *in vitro* followed by HVPE was used in attempts to detect these intermediates. Cultures of Stonier's medium (250 ml) were inoculated with strains NT1 and K439. Cells were grown, harvested and sonicated as described in section 2.18. Cell-free extracts were mixed with agrocinopine B purified by paper electrophoresis. The reactions were either stopped immediately on ice by addition of acetic acid or incubated at 28°C for two h before stopping the reaction with acetic acid. After centrifugation to remove precipitated proteins, the supernatants were subjected to HVPE in ammonium borate buffer to visualize the reaction products. Ammonium borate was used because neutral sugars are separable in this buffer (71). Fig. 9 shows an electrophoretogram of such an experiment, indicating that over a two h incubation period, an extract from strain NT1 had no visible effect on agrocinopine B, while an extract of strain K439 converted agrocinopine B to a compound with a slightly faster electrophoretic mobility. However, despite repeated attempts using the same two strains, this result could not be repeated.

Another method to allow cellular catabolic enzymes ready access to the opine substrate was tried. Cells were made permeable by toluenization prior to addition of opine as described in section 2.18. Toluened cells of strain K439 had no visible effect on the opine substrate. Since silver
Figure 9. Catabolism *in vitro* of agrocinopine B using cell-free extracts of *Agrobacterium* strains NT1 and K439. Samples removed from cell-free extract reactions were stopped with acetic acid and centrifuged. The superatants were subjected to HVPE ammonium borate buffer. The borate was removed by dipping the paper in a sulfuric acid-ethanol solution, followed by alkaline silver nitrate staining (see sections 2.11 and 2.18 for details). The novel silver nitrate-positive compound is marked (>). Labels are as described in the legend to Fig. 7.
nitrate staining of papers electrophoresed in ammonium borate buffer is poor, we tried separation of samples by descending paper chromatography. Descending paper chromatography was not a useful technique for analysing agrocinopine breakdown, due to a large number of strongly silver nitrate-positive compounds on the chromatograms, which obscured the opine signal. A reason why the K439-associated alteration of agrocinopine B could not be repeated was never identified.

3.8. Insertional mutagenesis of the acc region.

In order to more precisely map acc, and to analyze acc transcription, we mutagenized the region with transposon Tn3-HoHol (165). This Tn3 derivative contains a promoterless lacZ gene just inside the left inverted repeat. In addition to creating insertional mutations, Tn3-HoHol can form transcriptional and translational fusions between the lacZ gene and any gene into which the transposon inserts. β-galactosidase activity of these lacZ fusions can be quantitatively analyzed as a measure of transcriptional activity. pTHB112 was selected as the target plasmid, as it contains the agrocinopine-agrocin 84 region centrally positioned on its pTiC58 insert. Tn3-HoHol insertion derivatives of this clone were isolated in E. coli strain C2110 as described in section 2.8. Plasmid DNAs isolated from small (1.5 ml) cultures were analyzed by digestion with restriction enzymes BamHI, EcoRI, HindIII, and SmaI followed by agarose gel electrophoresis to map the transposon positions and orientations. Over 180 insertions were examined and 50 were found to be located on the insert fragment. Each of these plasmids was transformed into A. tumefaciens strain NT1, using carbenicillin as the selective antibiotic, and the progeny assayed for agrocin 84 sensitivity and β-galactosidase
activity. The locations, orientations and conferred phenotypes of 23 such 
\text{Tn}^\text{3}-\text{HoHol} mutations are shown in Fig. 10. All insertion mutants that were 
tested against agrocin 84 showed only one of two phenotypes; agrocin 84 
resistance or agrocin 84 sensitivity inducible by agrocinopines A+B. No 
mutants were isolated which had an agrocin 84-supersensitive, noninducible 
phenotype. All insertions abolishing agrocin 84 sensitivity mapped to a 
5.1 kb region. Insertions outside of this region continued to confer an 
opine-inducible agrocin 84 phenotype. Indicator plates containing the 
chromogenic $\beta$-galactosidase substrate X-gal showed that only those inserts 
in \text{acc} in which the \text{lacZ} gene is transcribed from left to right [clockwise 
on the map of Depicker et al. (41)] produce $\beta$-galactosidase (Fig. 10). 
These insertion plasmids conferred the identical $\beta$-gal phenotypes shown 
in Fig. 10 on \text{E. coli}, indicating that \text{acc} is transcribed and translated 
in this organism. Analysis of the insertions indicated that the left end 
of \text{acc} must lie between the left end of \text{HindIII} fragment 31a and insertion 
476. The right end of \text{acc} lies between the right end of \text{BamHI} fragment 
5 and insertion 587 (see box in Fig. 10).

The effect of the \text{Tn}^\text{3}-\text{HoHol} insertions on agrocinopine uptake by 
strain NT1 was analyzed by HVPE. Only NT1(pTHB478) and NT1(pTHB587) 
removed detectable amounts of agrocinopines A+B from the medium (Fig. 11). 
These clones are the only plasmids of the seven tested that still confer 
agrocin 84 sensitivity. Uptake experiments with six of the same insertion 
mutants [NT1(pTHB587) was not tested] using lower (1.5 mM) agrocinopine 
concentrations and 50-fold higher cell concentrations yielded identical 
results.
Figure 10. Tn3-HoHol insertion map of pTHB112. Tn3-HoHol insertions were constructed and mapped as described in Materials and Methods. Restriction map and transposon locations are revised from those previously published (73). Response to agrocin 84 (S, sensitive and agrocinopine-inducible; R, resistant) was determined by testing strain NT1 containing each insertion plasmid by the plate bioassay. Crossbars on each insertion indicate transcriptional orientation of the Tn3-HoHol lacZ gene. β-galactosidase activity, as determined on X-gal indicator plates, was assigned a +, ±, or - value. +, blue; ±, light blue; -, white or very light blue. Each Tn3-HoHol derivative has the prefix pTHB-. Relevant cosmid clones defining the region are shown along with their agrocin 84 phenotypes at the bottom of the figure. Coordinates (in kb) refer to the map of pTiC58 (41).
Figure 11. HVPE analysis of agrocinopine uptake by strain NT1 containing selected pTHB112::Tn3-HoHol derivatives. Culturing, electrophoresis, staining and abbreviations are as described in the legend to Fig. 7. Each construct shown in bold characters has the prefix pTHB- (see Fig. 10). Incubation times are in hours. Strain NT1(pTHB587) gave results similar to strain NT1(pTHB478), and strain NT1(pTHB566) gave results similar to strain NT1(pTHB422).
In summary, the correlation between agrocin 46 sensitivity and agrocinopine transport has been strengthened. Each and every mutation that ablated agrocin sensitivity also inactivated agrocinopine transport. No mutation was found that separated the two phenotypes. Transposon mutagenesis was used to more accurately determine the size of the region. It was found to be between 5.1 and 7.2 kb in length. No mutation was found in the uninducible phenotype.

Specifically, we wished to determine whether the inducing effect of agrocinopines Ap on Acp is at the transcriptional level. Experiments described in a later section (3.12) characterized the regulatory function itself. The Inl::Bacterial gal::lacZ fusions allowed us to measure the transcriptional activity of the region, and to quantitate its induction by agrocinopines. B-galactosidase activity was measured in the presence and absence of inducing amounts of agrocinopines. In A. tumefaciens strains containing four different plasmids. The strains were grown at 28°C in Stocker's medium (6 ml) containing tercatocycline (1 mg/ml). At an O.D. of 0.3 to 0.5, the cultures were split into two 1 ml subcultures in Erlenmeyer flasks. A chloroform-sterilized solution of agrocinopines Ap was added to one culture of each pair, at a final concentration of
In summary, the correlation between agrocin 84 sensitivity and agrocinopine transport has been strengthened. Each and every mutation that abolished agrocin sensitivity also inactivated agrocinopine transport. No mutation was found that separated the two phenotypes. Transposon mutagenesis was used to more accurately determine the size of \( \text{acc} \); it was found to be between 5.1 and 7.2 kb in length. No mutation was found that yielded an agrocin 84-supersensitive, noninducible phenotype, indicating that the regulatory locus does not appear to be inactivated by any of the insertions. Although the \( \text{acc} \) region does not confer agrocin 84 sensitivity or agrocinopine uptake on \( \text{E. coli} \), the transcriptional activity of the region in this organism appears similar to that in \( \text{Agrobacterium} \).

3.9. Induction by agrocinopines.

We were interested in characterizing the regulation of the \( \text{acc} \) region. Specifically, we wished to determine whether the inducing effect of agrocinopines A+B on \( \text{acc} \) is at the transcriptional level. Experiments described in a later section (3.12) characterized the regulatory function itself. The Tn\(^3\)-HoHol \( \text{acc}::\text{lacZ} \) fusions allowed us to measure the transcriptional activity of the region, and to quantitate its induction by agrocinopines. \( \beta \)-galactosidase activity was measured in the presence and absence of inducing amounts of agrocinopines, in \( \text{A. tumefaciens} \) strains containing four different plasmids. The strains were grown at 28°C in Stonier's medium (8 ml) containing tetracycline (1 \( \mu \)g/ml). At an O.D. \( _{600} \) of 0.3 to 0.5, the cultures were split into two 4 ml cultures in 125 ml flasks. A chloroform-sterilized solution of agrocinopines A+B was added to one culture of each pair, at a final concentration of
approximately 25 µM, and all cultures were reincubated as described above. Samples (0.1 ml) were removed from the cultures at two h intervals and assayed for β-galactosidase activity as described in section 2.9. Values obtained were proportional to units of β-galactosidase activity per cell per min. The O.D.₀₆₀₀ of the cultures at each sampling time was measured by aseptically transferring the cells to a sterile 100 X 75 mm glass tube, measuring the optical density, and returning the culture to the 125 ml flask for continued incubation at 28°C on a shaker.

Two ace::lacZ fusions were used to measure acc transcriptional activity. pTHB476 has a β-Gal⁻ Tn3-HoHol insertion in the left end of the region that abolishes agrocin 84 sensitivity. pTHB555 contains a Tn3-HoHol insertion into the right end of the region that is β-Gal⁺ and also eliminates agrocin 84 sensitivity. Tn3-HoHol is inserted outside and to the left of acc in pTHB578, where it has no effect on agrocin 84 sensitivity or agrocinopine transport, and is β-Gal⁻ (Figs. 10, 11). The unmutated cosmid clone pTHB112 was also included as a negative control. Fig. 12A shows the results of a representative one of two experiments testing the effect of agrocinopines on β-galactosidase activity of acc::lacZ fusion 476 in Ti-plasmidless strain NT1. Agrocinopines had no effect on β-galactosidase levels produced by fusion 476 in this host. This result led us to recall that transposon insertions 476 and 555 eliminate acc function, including agrocinopine transport (Figs. 10, 11). If the opine cannot enter the cell, it should not be able to influence acc expression. It was therefore necessary to assay these lacZ fusions in a strain such as K439, which contains a second, functional, acc-encoded uptake system. β-galactosidase levels in the uninduced left-end mutant
Figure 12. Induction of β-galactosidase by agrocinopines A+B in strains NT1 and K439 containing recombinant plasmids. Strains, grown to mid-exponential phase in Stonier's medium containing 1 µg/ml tetracycline, were harvested and then washed with and resuspended in an equal volume of Stonier's medium. A sample of agrocinopines A+B was chloroform-sterilized and added to a final concentration of 25 µM (solid symbols) at zero h. Portions were removed and assayed for β-galactosidase activity at the times indicated. Graphs A and B each show the results of a representative one of two experiments. A. ▼, NT1(pTHB112); Δ, ▲, NT1(pTHB476). B. ▼, K439(pTHB112); Δ, ▲, K439(pTHB476); □, ■, K439(pTHB555); ○, ●, K439(pTHB578).
K439(pTHB476) were 20- to 30-fold greater than in strain K439 containing either of the β-Gal− plasmids pTHB578 or pTHB112. Similarly, uninduced β-galactosidase levels in the right-end mutant K439(pTHB555) were approximately 4- to 6-fold higher than in strain K439 containing the β-Gal− recombinant plasmids (Fig. 12B). No effect of the opines on β-galactosidase levels in strain K439(pTHB578) was detected. Agrocinopines A+B caused a 3- to 6-fold increase in the β-galactosidase activity of strain K439(pTHB476) and a 4- to 5-fold increase in enzyme activity in strain K439(pTHB555) by 2 to 6 h after their addition (Fig. 12B). Since E. coli does not take up agrocinopines even when harboring a functional acc region (Fig. 7B), agrocinopine induction of acc::lacZ fusions was not tested in this host.

To summarize, β-galactosidase activity in Agrobacterium strains containing acc::lacZ fusions in either end of the region is induced by agrocinopines. This and the observation that only Tn3-HoHo1 insertions transcriptionally oriented from left to right within the region are β-Gal+ (Fig. 10) are consistent with acc being composed of a single transcriptional unit (73). Both acc::lacZ fusion strains tested show uninduced β-galactosidase levels well above background, indicating that the region is transcribed in the absence of inducer opines. This is consistent with the sensitivity of strain C58 to agrocin 84 in the absence of the opine inducer. Induction is dependent on a host strain containing a second, functional acc region, presumably to allow the inducing opines entry into the cell. Agrocinopines have no effect on the basal β-galactosidase activity of a strain containing a Tn3-HoHo1 insertion outside of acc.
3.10. **Complementation analysis of the acc region.**

If the acc region consists of a single transcriptional unit, it should not be possible to complement *in trans* Tn3-HoHol insertions within acc using subcloned fragments containing portions of the region. Transposon insertions would have polar effects on all genes downstream, and subcloned fragments would lack either the 5' promoter or 3' sequences. To test this, previously constructed overlapping subclones (see section 3.4) were assayed for their ability to restore acc function when placed *in trans* to mutations within the region. None of these subclones or transposon insertion plasmids by themselves conferred agrocin 84 sensitivity (ref. 73, Figs. 6 and 10 this work). A series of merodiploids were constructed by introducing the subclones into *Agrobacterium* strains containing Tn3-HoHol insertion plasmids. In initial experiments, merodiploids were constructed and tested for complementation in the Rec+ strain, NT1. Concomittant plasmid isolation and electrophoretic analyses were performed to ensure that no rearrangements or recombination events were occurring. In subsequent experiments, the NT1 recA strain UIA143 was used to prevent homologous recombination between the two plasmids. Each merodiploid strain was tested for sensitivity to agrocin 84 in the plate bioassay, as well as for the presence of both plasmids. Pairs of subcloned fragments were also tested for their ability to reconstitute acc. Results of these analyses are shown in Fig. 13A. Restriction fragment EcoRI 26 failed to complement either 476 or 426, the two insertion mutations that it overlaps. However, BamHI 5, which extends further to the left and right, complemented both of these mutations. HindIII fragment 11 did not complement insertion 426, but did complement
Figure 13. Complementation analysis of the \textit{ace} region. A. Restriction map of \textit{ace}-containing cosmid clone pTHB112 showing Tn$3$-HoHol insertion positions and complementation data. Subcloned fragments shown, cloned into pSa152 (169), were introduced into strain UIA143 containing Tn$3$-HoHol insertion derivatives of pTHB112. Tn$3$-HoHol insertions are described in the legend to Fig. 10. Flags at the top of the posts indicate transcriptional direction of \textit{lacZ}. Agrocin 84 sensitivity or resistance of merodiploids, determined by plate bioassay, is indicated by a + or - beneath each insertion for each of the five subcloned fragments tested. Brackets indicate two subcloned fragments tested in the same strain. B. Agrocin 84 bioassay plates were centrally inoculated with strain NT1(pAgK84-A1), incubated two days at 28°C, overlaid with different strains, and incubated two days at 28°C. Chloroform-sterilized paper strips containing 10 nmol agrocinopines A+B were placed on the overlays prior to incubation. For 1 through 4, each plate is representative for all positive complementation assays using the indicated subclone. Overlaid strains are 1; UIA143(pTHB476, pTHS9), 2; UIA143(pTHB476, pTHS5), 3; UIA143(pTHB566, pTHS11), 4 and 5; NT1(pTHB566, pTHS16). Plate 5 was not inoculated with strain NT1(pAgK84-A1) and therefore contains no agrocin 84. A chloroform-sterilized paper disc containing 20 nmol agrocinopines A+B was placed on the overlay prior to incubation.
### A

**AGROCIN**

<table>
<thead>
<tr>
<th>BamHI</th>
<th>14</th>
<th>5</th>
<th>17</th>
<th>7</th>
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<tr>
<td>HindIII</td>
<td>3</td>
<td>42</td>
<td>31a</td>
<td>11</td>
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<tr>
<td>EcoRI</td>
<td>33</td>
<td>21</td>
<td>26</td>
<td>16</td>
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<td>SmaI</td>
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<tr>
<th>kb</th>
<th></th>
<th>130</th>
<th></th>
<th>140</th>
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- Small DNA fragments are shown in the diagram.
- The upper strand contains agrocin phages and pTHSB28.
- The lower strand contains pTHSB5 and pTHSS9.

### B

[Image of bacterial plates with agar and antibiotic resistance tests]

- Plate 1: No growth
- Plate 2: Growth on antibiotic
- Plate 3: Growth on antibiotic
- Plate 4: No growth
- Plate 5: Growth on antibiotic

By hypothesis, the gene was transcribed from left to right as indicated by the direction of growth on the plates.
insertions 566 and 422. BamHI fragment 5 and EcoRI 16 also complemented mutations 566 and 422. BamHI fragment 5 did not complement insertion mutation 555, but HindIII 11 and EcoRI 16, which contain sequences to the right of BamHI 5, both complemented transposon insertion 555.

Fragments BamHI 5 and HindIII 11 reconstituted a functional region in trans to each other, while BamHI 5 did not complement EcoRI 16 (Fig. 13A), presumably because neither of these two fragments contains the right-hand end of the region.

Agrocin 84 sensitivity of merodiploids containing any one of the five Tn3-HoHol insertion plasmids complemented with subcloned fragments HindIII 11 or SmaI 9 was not discernably enhanced in the vicinity of the paper strip containing agrocinopines A+B, placed radially on the plate (Fig. 13B). On the other hand, analysis of merodiploid strains containing either subcloned EcoRI 16 or BamHI 5 in trans to any of the Tn3-HoHol insertions showed a zone of inhibition extending completely around the agrocinopine strip. This raised the possibility that these strains were sensitive to agrocinopines A+B as well as to agrocin 84. To test this possibility, Stonier's medium plates containing no agrocin 84 were overlaid with these merodiploid strains and paper discs impregnated with agrocinopines A+B placed on top of the overlays. Following incubation at 28°C for 24 h, growth of the strains was found to be inhibited by the opines. This inhibitory effect was most pronounced in merodiploids containing EcoRI 16 (Fig. 13B).

These complementation results were inconsistent with our previous hypothesis that acc was transcribed from left to right as a single unit (73). If this were true, how could subcloned fragments such as EcoRI 16
and HindIII 11, which should lack promoter sequences for the postulated transcript, rescue Tn3-HoHol insertional mutations that abolish agrocin 84 sensitivity? To help answer this question, complementation groups were further defined by first generating another set of mutations in ace. These were then tested in complementation analyses with Tn3-HoHol insertion mutants. This second group of mutants was obtained by mutagenizing the ace subclone pTHH206 (Agr\(^{+}\) Acp\(^{-}\)) with Tn5 as described in section 2.8. Over 60 insertions into pTHH206 were mapped using the restriction enzymes HindIII, BamHI and SmaI. All of the Tn5 insertions tested in pTHH206 abolished agrocin 84 sensitivity in strain UIA143 except insertion 313. This plasmid conferred agrocin 84 sensitivity not inducible by agrocinopines (Fig. 14B). The left end of the ace region must therefore lie between Tn5 insertions 313 and 352. The right end lies between the right end of BamHI fragment 5 and Tn3-HoHol insertion 587 (Fig. 14A).

Selected ace::Tn5 insertion mutations were then tested for their ability to restore agrocin 84 sensitivity when in trans to pTHB112::Tn3-HoHol ace insertion plasmids (Fig. 14A). Three Tn5 insertions mapped to the right of insertion 313, and in the same area as Tn3-HoHol insertions 476 and 426. These three Tn5 insertions, 352, 301, and 349, complemented all Tn3-HoHol insertions except 476 and 426. Further to the right, within EcoRI fragment 16, Tn3-HoHol insertions 566 and 422 were found to behave differently with respect to Tn5 insertions 312, 306, and 304. Insertion 312 complemented four of the five Tn3-HoHol insertions, including 422, but failed to rescue insertion 566. Similarly, Tn5 insertions 306 and 304 complemented all Tn3-HoHol insertions except 422. The two Tn5 insertions
Figure 14. Complementation analysis of merodiploids containing Tn3-HoHol and Tn5 insertion plasmids. A. Agrocin 84 sensitivity or resistance of merodiploids, determined by plate bioassay, is indicated by a + or - beneath each Tn3-HoHol insertion and above the line representing each Tn5 derivative. Tn5 insertion positions are marked (▼) and plasmid names are given at the left. Agrocin 84 reactions for each Tn5 derivative alone in strain UIA143 are given to the right. The box at the top of the figure indicates the minimum size of the acc region (shaded) and its maximum limits (open boxes). B. Plate bioassays of merodiploid strains containing pTHH317, and of strain UIA143(pTHH313). Overlaid strains (UIA143) contain the following plasmids: 1, pTHH313; 2, pTHB476 + pTHH317; 3, pTHB426 + pTHH317; 4, pTHB566 + pTHH317; 5, pTHB422 + pTHH317; 6, pTHB555 + pTHH317; 7, pTHH317.
### A

<table>
<thead>
<tr>
<th></th>
<th>BamHI</th>
<th>HindIII</th>
<th>EcoRI</th>
<th>Smal</th>
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<tr>
<td></td>
<td>14</td>
<td>3-42</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>5</td>
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#### Agrocin

<table>
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<th>140</th>
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<tr>
<td>pTHH313</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH352</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH301</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH349</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pTHH312</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH306</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH304</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH348</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pTHH314</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>pTHH317</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

#### B

![Image of agar plates](image-url)
348 and 314, mapping within SmaI fragment 22, each complemented all five of the Tn3-HoHol insertions. Lastly, Tn5 insertion 317 and Tn3-HoHol insertion 555 map very close together at the right end of the region. The latter is the only Tn3-HoHol mutation that Tn5 insertion 317 did not complement (Fig. 14B).

To summarize, complementation analyses using paired transposon insertions into the acc region have helped to clarify ambiguities generated by complementation tests using subcloned fragments of acc. Rather than a simple locus consisting of a single transcript, the region appears more complex, probably encoding several transcripts. Its size has been more accurately determined to be from 5.3 to 6.9 kb.

3.11. Agrocin 84 transport

To extend the correlation between agrocin 84 sensitivity and agrocinopine transport to include agrocin 84 transport, we developed a plate bioassay that detects removal of agrocin 84 from liquid cultures by concentrated cell suspensions. Strains were grown at 28°C in Stonier’s medium to exponential phase and concentrated fifty-fold, mixed with partially purified samples of agrocin 84 in 30 µl cultures, and incubated at 28°C on a shaker. Samples were withdrawn at selected intervals, the cells removed by centrifugation, and the supernatants assayed for the presence of the antibiotic as follows. The supernatants were spotted on Stonier’s medium plates and allowed to absorb into the agar. After chloroform-sterilization, the plates were overlaid with top agar seeded with agrocin 84-sensitive indicator strains K439 or NT1(pTiC58Tra5) and incubated at 28°C. A decrease in inhibition zone size in plates spotted
with culture samples removed at later times was taken as an indication of agrocin 84 accumulation by the cells.

Strains C58, K439, and NT1 were tested for their ability to take up partially purified agrocin 84 in this manner. As shown in Fig. 15, strain NT1 (column C) did not remove detectable amounts of agrocin 84 from the medium after 4 h, and the inhibition zone was only slightly smaller at 24 h. Consistent with previously published results (51), strain K439 (column B) took up agrocin 84 so rapidly that almost immediately upon mixing, most of the antibiotic had disappeared from the culture medium. Almost all of the agrocin 84 was removed from the medium by 4 h. Again consistent with previous observations (51), strain C58 did not take up the antibiotic as rapidly as strain K439. Strain C58 required 24 h to remove the same amount of agrocin 84 that strain K439 took up in 4 h (column A).

The cosmid clones shown in Figs. 3 and 4, and subclone pTHH206 were tested in strain NT1 for their ability to confer agrocin 84 uptake. pTHB112, pTHH32 and subclone pTHH206, which encode agrocin 84 sensitivity and agrocinopine transport, conferred uptake of agrocin 84 as measured by this assay (Table 3). Cosmid clones pTHB55 and pTHE16, which overlap acc but do not confer agrocin 84 sensitivity, failed to allow transport of the antibiotic by the NT1 host strain (Table 3). In a similar fashion, Tn3-HoHol derivatives shown in Fig. 10 were tested in strain NT1 to determine the effect of the insertions in acc on the transport of the antibiotic. Only those constructs that conferred agrocin 84 sensitivity and agrocinopine uptake also allowed transport of agrocin 84 (Fig. 16). Agrocin 84 uptake by E. coli containing acc plasmids was not tested.
Figure 15. Agrocin 84 uptake by *Agrobacterium* strains. Following growth to mid-exponential phase in Stonier's medium, cultures were concentrated 50-fold and incubated with partially purified agrocin 84 as described in section 2.15. Samples (10 µl) were removed at the hour time intervals shown at the right, centrifuged and the supernatants spotted on Stonier's plates. The indicator strain used to inoculate the overlays was K439. A, C58 (Agr'); B, K439 (Agr<sup>ss</sup>); C, NT1 (Agr').
Table 3. Agrocin 84 transport by *Agrobacterium* strains containing *acc* clones.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transport</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT1</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td>C58</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>K439</td>
<td>+</td>
<td>SS</td>
</tr>
<tr>
<td>NT1(pTHB112)</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>NT1(pTHH32)</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>NT1(pTHB55)</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td>NT1(pTHE16)</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td>NT1(pTHH206)</td>
<td>+</td>
<td>S</td>
</tr>
</tbody>
</table>

* Determined by mixing concentrated cell suspensions of the indicated strains with partially purified agrocin 84 and assaying for the presence of the antibiotic in the culture supernatant, as described in Chapter II.

* +, all detectable agrocin 84 removed from culture medium by 24 h; -, little or no decrease in detectable amount of agrocin 84 in culture medium by 24 h.

* R, agrocin 84-resistant; S, agrocin 84-sensitive; SS, agrocin 84-supersensitive or constitutive.
Figure 16. Agrocin 84 uptake by strain NT1 containing pTHB112::Tn3-HoHol derivatives. Assays were performed as described in the legend to Fig. 15. Hours of incubation with agrocin 84 are shown at the right. Strains tested were: A, C58 (Agr'); B, NT1 (Agr'); C, NT1(pTHB478) (Agr'); D, NT1(pTHB476) (Agr'); E, NT1(pTHB426) (Agr'); F, NT1(pTHB566) (Agr'); G, NT1(pTHB422) (Agr'); H, NT1(pTHB587) (Agr').
The pattern established with agrocin 84 sensitivity and agrocinopine transport has been extended to include agrocin 84 uptake. Only those recombinant plasmids shown to confer the first two phenotypes confer the third. All plasmids analyzed that failed to confer any one phenotype also failed to confer the other two; all three phenotypes are genetically inseparable.

3.12. **Locating the acc-tra regulatory function.**

Strains K439 and NT1(pTiC58Tra^+) are supersensitive to agrocin 84 presumably because they possess a defective negative acc regulator (51). We theorized that introducing a cloned restriction fragment encoding the wild type regulator into these strains would return their constitutive agrocin 84-supersensitive phenotype to one that, like strain C58, is inducible by agrocinopines. Different cosmid clones of the acc region (see Fig. 4) were introduced into strain K439, and these merodiploid strains were examined for opine-inducibility using the plate bioassay (Table 4). Clones pTHB58, pTHE16, and pTHH9 had no effect on the agrocinopine-uninducible agrocin 84-supersensitive phenotype of strain K439. Plasmids pTHB112 and pTHH32 altered the phenotype of strain K439 so that it was slightly inducible by agrocinopines. Strain K439 containing pTHB55 was sensitive to agrocin 84, and that sensitivity was fully inducible by agrocinopines. pTHH7 greatly reduced the agrocin 84 sensitive phenotype of strain K439. This reduced sensitivity was slightly induced by agrocinopines A+B (Table 4). These results indicate that the regulatory function maps somewhere on BamHI fragments 14 or 5, probably to the left of HindIII 11 (Fig. 4).
Table 4. Effect of acc cosmid clones, Tn3-HoHo1 insertion plasmids, and cloned restriction fragments on agrocin 84 sensitivity conferred by pTiC58 and pTiC58Tra^c.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source of clone</th>
<th>Agrocin 84 phenotype^a - agrocinopines</th>
<th>+ agrocinopines</th>
</tr>
</thead>
<tbody>
<tr>
<td>UIA143(pTiC58)</td>
<td></td>
<td>S</td>
<td>SS</td>
</tr>
<tr>
<td>C58</td>
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<td>S</td>
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<tr>
<td>UIA143(pTiC58Tra^c)</td>
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<td>SS</td>
<td>SS</td>
</tr>
<tr>
<td>K439</td>
<td></td>
<td>SS</td>
<td>SS</td>
</tr>
<tr>
<td>UIA143</td>
<td></td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>NT1</td>
<td></td>
<td>R</td>
<td>R</td>
</tr>
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<td>SS</td>
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<td>SS</td>
</tr>
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<td>SS</td>
<td>SS</td>
</tr>
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</tr>
<tr>
<td>K439(pTHB426)</td>
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<td>S**</td>
</tr>
<tr>
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<td>S**</td>
</tr>
<tr>
<td>K439(pTHB555)</td>
<td>pTiC58</td>
<td>S*</td>
<td>S**</td>
</tr>
<tr>
<td>K439(pTHSE26)</td>
<td>pTiC58</td>
<td>R</td>
<td>R</td>
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Table 4. Continued.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source of clone</th>
<th>- agrocinopines</th>
<th>+ agrocinopines</th>
</tr>
</thead>
<tbody>
<tr>
<td>K439(pTHVH31)</td>
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<td>R</td>
<td>R</td>
</tr>
<tr>
<td>UIA143(pTiC58,Tra&lt;sup&gt;c&lt;/sup&gt;,pTHSE26)</td>
<td>pTiC58</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>UIA143(pTiC58,Tra&lt;sup&gt;c&lt;/sup&gt;,pTHSB5)</td>
<td>pTiC58</td>
<td>S</td>
<td>SS</td>
</tr>
<tr>
<td>UIA143(pTiC58,Tra&lt;sup&gt;c&lt;/sup&gt;,pSVKE26)</td>
<td>pTiC58&lt;sup&gt;Tra&lt;sup&gt;c&lt;/sup&gt;&lt;/sup&gt;</td>
<td>SS</td>
<td>SS</td>
</tr>
<tr>
<td>UIA143(pTiC58,Tra&lt;sup&gt;c&lt;/sup&gt;,pSVKB5)</td>
<td>pTiC58&lt;sup&gt;Tra&lt;sup&gt;c&lt;/sup&gt;&lt;/sup&gt;</td>
<td>SS</td>
<td>SS</td>
</tr>
<tr>
<td>UIA143(pTiC58,pTHSE26)</td>
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<td>LS</td>
<td>S</td>
</tr>
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<td>UIA143(pTiC58,pTHSB5)</td>
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<td>S</td>
<td>SS</td>
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<tr>
<td>UIA143(pTiC58,pSVKE26)</td>
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<td>S</td>
<td>SS</td>
</tr>
<tr>
<td>UIA143(pTiC58,pSVKB5)</td>
<td>pTiC58&lt;sup&gt;Tra&lt;sup&gt;c&lt;/sup&gt;&lt;/sup&gt;</td>
<td>S</td>
<td>SS</td>
</tr>
</tbody>
</table>

* Determined in the plate bioassay described in Materials and Methods. S, sensitive (wild-type); SS, supersensitive or constitutive; LS, less sensitive than wild-type; R, resistant; S*, reduced sensitivity that varies from experiment to experiment; S**, inducing effect of agrocinopines on S* phenotype, also variable; SS*, supersensitive with slight inducing effect of agrocinopines.
To determine whether any of the Tn3-HoHol insertions had inactivated this trans-acting regulatory function, selected Tn3-HoHol insertion plasmids were introduced into strain K439 and the strains tested for agrocin 84 sensitivity. Those plasmids with insertions within acc conferred a reduced agrocin 84-sensitive phenotype on strain K439 that was variable from insertion to insertion, and from experiment to experiment with the same insertion. Agrocinopines had an inducing effect that was also variable (Table 4). pTHB478, whose Tn3-HoHol insertion lies to the left of acc (see Fig. 10), conferred an agrocin 84-supersensitive, slightly agrocinopine-inducible phenotype on strain K439, similar to that of K439(pTHH32). When different subcloned fragments of the acc region were tested in this manner, BamHI fragment 5, which encodes all but the right end of acc, was found to confer an agrocinopine-inducible agrocin 84-sensitive phenotype on the constitutive strain UIA143(pTiC58Tra'). Fragment EcoRI 26, encoding only the left end of acc and contained within BamHI 5, had an even stronger effect, causing the strain to become resistant to agrocin 84. HindIII fragment 31a, nested within EcoRI 26, had the same effect (Table 4). Similar effects were observed with cloned BamHI 5 and EcoRI 26 fragments in trans to the wild-type Ti plasmid pTiC58 in strain UIA143 (HindIII 31a was not tested). Fragments BamHI 5 and EcoRI 26, cloned from the transfer-constitutive Ti plasmid (S. Beck Von Bodman, personal communication) had no effect on agrocin 84 sensitivity when introduced into strain UIA143 carrying either the wild-type or mutant Ti plasmid (Table 4).

Since the cloned wild-type EcoRI 26 fragment had such a marked negative effect on agrocin 84 sensitivity, it was further analysed for
effects on other acc phenotypes. The previously described agrocin 84 uptake assay was used to determine the effect of fragment EcoRI 26 on transport of the antibiotic. In this assay, pTiC58Tra^ conferred the ability to rapidly remove agrocin 84 from the culture medium (Fig. 15, column B and Fig. 17A, column 1). Introduction of pTHSE26, which contains EcoRI 26, into such acc-constitutive strains reduced or abolished this ability (Fig. 17A, column 4). Subcloned HindIII 31a had a similar, but somewhat lesser, effect (column 5). The EcoRI 26 subclone also appeared to slow the removal of agrocinopines from culture media (Fig. 17B).

The effect of EcoRI fragment 26 on transcription of the region was determined in E. coli and Agrobacterium strains containing acc::lacZ fusion plasmids pTHB476 or pTHB426. The β-galactosidase activities of the E. coli merodiploids were reduced by approximately 30 to 50% compared to the strains containing pTHB476 or pTHB426 and vector pSal52 (Table 5). In Agrobacterium, introduction of the wild-type EcoRI 26 subclone pTHSE26 into a strain containing acc::lacZ fusion plasmid pTHB476 also reduced β-galactosidase activity by approximately 30% (Table 5).

Finally, the ability of plasmid pTHSE26 to negatively affect Ti plasmid conjugal transfer was tested, employing the drop method described in section 2.7. Ti plasmid transfer matings were performed using strains C58, NT1(pTiC58Tra^), and NT1(pTiC58Tra^) containing subclone pTHSE26 or vector pSal52 as donor strains. Donors, grown in ATN supplemented with mannitol and containing kanamycin, were spotted on lawns of C58ClRS spread on ATN minimal medium plates containing nopaline (2 mg/ml) to select for the transfer of the Ti plasmid, and rifampicin and streptomycin to select against the donor strains. pTiC58Tra^ transferred at a frequency of 1.2
Figure 17. Effect of cloned fragment EcoRI 26 on pTiC58Tra⁺-mediated agrocin 84 and agrocinopine transport. A. Agrocin uptake assays were performed as described in the legend to Fig. 15. Incubation times in hours are at the right. Strains analyzed were: 1, UIA143(pTiC58Tra⁺); 2, UIA143; 3, UIA143(pTiC58); 4, UIA143(pTiC58Tra⁺, pTHSE26); 5, UIA143(pTiC58Tra⁺, pTHVH31). B. HVPE analysis of agrocinopine transport by strains K439 and K439(pTHSE26). Labels are described in the legend to Fig. 7.
Table 5. Effect of cloned EcoRI fragment 26 on acc transcription.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \beta )-galactosidase units (units/cell/min.)\textsuperscript{b}</th>
<th>( \Delta \beta )-galactosidase activity\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. ) coli</td>
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<td></td>
</tr>
<tr>
<td>DHl(pTHB476,pSal52)</td>
<td>947.0</td>
<td>34.1%</td>
</tr>
<tr>
<td>DHl(pTHB476,pTHSE26)</td>
<td>624.3</td>
<td>52.0%</td>
</tr>
<tr>
<td>DHl(pTHB426,pSal52)</td>
<td>1242.7</td>
<td></td>
</tr>
<tr>
<td>DHl(pTHB426,pTHSE26)</td>
<td>596.1</td>
<td></td>
</tr>
<tr>
<td>( A. ) tumefaciens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTl(pTHSE26)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>NTl(pTHB476)</td>
<td>122.5</td>
<td>29.9%</td>
</tr>
<tr>
<td>NTl(pTHB476,pTHSE26)</td>
<td>85.9</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Shown are representative values from one of two experiments.

\textsuperscript{b} Measured using the quantitative assay of Miller (128) as described in section 2.9.

\textsuperscript{c} Calculated as 1 minus the ratio of the \( \beta \)-galactosidase activity of the acc-lac\( Z \) strain containing subcloned EcoRI 26 divided by the \( \beta \)-galactosidase activity of the corresponding strain lacking the subcloned fragment, multiplied by 100.
$10^{-3}$ transconjugants/input donor (Table 6). The transfer frequency of this Trac mutant was at least five orders of magnitude higher than the wild-type parental strain C58. When pTHSE26 was introduced into the donor strain, the transfer frequency of the Ti plasmid was reduced approximately 100-fold (Table 6). The subcloned EcoRI 26 fragment isolated from the Trac Ti plasmid, when placed in trans to pTiC58Trac, had no such repressive effect (S. Beck Von Bodman, personal communication). The vector used to construct pTHSE26, pSal52, when present in strain NT1(pTiC58Trac), had little effect on the Ti plasmid transfer frequency (Table 6).

Since the tra-acc regulatory function appears to be encoded by EcoRI fragment 26, it is plausible that one of two Tn3-HoHol insertions mapping to this fragment might have inactivated it. This would indicate that the function is contained within the far left complementation group. Insertion 476 is the leftmost Tn3-HoHol insertion abolishing acc function, located in the center of EcoRI 26 (Fig. 10). To ascertain whether this insertion is within the regulatory gene, 476 was homogenotized into pTiC58 using pPH1JI as detailed in section 2.10. The Ti plasmid containing the transposon within EcoRI fragment 26 was then tested for its conjugal transfer frequency. If the regulatory function has been abolished by the transposon insertion recombined into the Ti plasmid, transfer should occur at a derepressed level. Such a homogenate should have the phenotypes Cb', Gm', Tc', Agr', Noc', and B-Gal'. Insertion 476 maps within restriction fragment BamHI 5, and this fragment is easily discernable in the restriction pattern when BamHI-digested pTiC58 DNA fragments are separated by electrophoresis. In an electrophoretic separation of BamHI digestion products of plasmid DNA from this homogenate, fragment BamHI 5 should be
Table 6. Effect of cloned EcoRI fragment 26 on Ti plasmid conjugal transfer.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transconjugants/input donor(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C58</td>
<td>&lt;5 \times 10^{-8}</td>
</tr>
<tr>
<td>NT1</td>
<td>&lt;4.3 \times 10^{-9}</td>
</tr>
<tr>
<td>NT1(pTiC58\text{Tra}^c)</td>
<td>1.2 \times 10^{-3}</td>
</tr>
<tr>
<td>NT1(pTiC58\text{Tra}^c,pTHSE26)</td>
<td>2.6 \times 10^{-5}</td>
</tr>
<tr>
<td>NT1(pTiC58\text{Tra}^c,pSal52)</td>
<td>5.2 \times 10^{-4}</td>
</tr>
</tbody>
</table>

\(^a\) Determined using the drop method mating (47) between the indicated donor strains and recipient strain C58ClRS as described in section 2.7. Representative values from one of two experiments are shown.
cleaved into two fragments of different mobilities, as Tn3-HoHol contains a single BamHI site. This should generate two new fragments approximately 6.6 and 15.8 kb in size. Colonies resistant to carbenicillin and gentamicin from the mating were tested and found to have lost tetracycline resistance and agrocin 84 sensitivity, were β-Gal-, and grew on ATN plates containing 2 mg nopaline/ml as the sole carbon and energy source. Ti plasmid DNA isolated from the strain into which insertion 476 had been homogenotized was digested with BamHI and subjected to gel electrophoresis along with BamHI-digested pTiC58. Fragment BamHI 5 was missing in the former pattern (Fig. 18). These seven traits together strongly imply that a double recombinational event has taken place between pTiC58 and pTHB476, with the loss of the cosmid vector, and the movement of Tn3-HoHol insertion 476 into the pTiC58 acc region. This strain, C58::476, when mated with C58ClRS, did not detectably transfer its Ti plasmid (less than 2 X 10^-7 transconjugants/input donor). Tra^ strain NT1(pTiC58Tra^) transferred its Ti plasmid at a frequency of 1.4 X 10^-3 transconjugants/input donor. Insertion 476 abolished both agrocin 84 sensitivity and agrocinopine transport (Figs. 10 and 11), and strain C58::476 was found to be resistant to agrocin 84. Insertion 476 does not result in constitutive transfer, and so is probably not located within the regulatory gene. We presumed that agrocinopines would be unable to enter this strain to induce conjugal transfer, and opine induction of conjugal transfer was therefore not tested.

In summary, we have isolated a 2.4 kb fragment that maps to the left end of the agrocin 84-agrocinopine region. This fragment encodes an activity that reduces or abolishes sensitivity to agrocin 84, reduces or
Figure 18. Agarose gel electrophoresis of \textit{Bam}HI-digested DNA from strain C58::476. Ethidium bromide-stained agarose gel of DNA samples electrophoresed in TBE buffer (section 2.5). Samples are: 1, plasmid DNA from strain C58::476; 2, pTiC58; 3, pTHB476. Size standards are indicated on the right in kb. The positions of the five largest \textit{Bam}HI digestion fragments of pTiC58 are shown on the left. The brightly-fluorescing bands in lane 1 are from pH4JI (79). Arrow (\textarrow) indicates the position of \textit{Bam}HI fragment 5.
Proteins from the cells were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. A protein of approximately 12 kDa (designated as P12) was synthesized by E. coli containing the wildtype plasmid at higher levels in the presence of IPTG. Approximately 12 kDa in size, this protein was synthesized by E. coli containing the lacZa::lacUV5 translational fusion plasmid at high levels in the presence of IPTG (Fig. 3).
abolishes transport of the antibiotic, appears to reduce agrocinopine transport, decreases acc transcription, and lowers Ti plasmid conjugal transfer frequencies at least two orders of magnitude (72). The analogous fragment isolated from pTiC58Tra' has no such negative effects on agrocin 84 sensitivity or conjugal transfer.

3.13. Proteins encoded by the acc region.

Given that the acc region appears to consist of several complementation groups, or genes, we were interested in determining the number and sizes of polypeptides synthesized by this region. To place acc fragments under the control of a regulatable promoter, we used the RSFl010-derived tac expression vectors pMMB22 and pMMB24 (6). Plasmids pTHE16 digested with BamHI and EcoRI, and pTHH205 digested with HindIII, were used as sources of inserts. The only fragment we were able to isolate containing part of acc was HindIII fragment 11 cloned in both orientations with respect to the IPTG-inducible tac promoter. pTHTH11A contains HindIII 11 with its acc transcriptional direction correctly oriented with respect to the tac promoter, while pTHTH11B contains the same fragment cloned in the reverse orientation. Cultures containing these two plasmids were grown in the presence of inducer IPTG as described in Chapter II, section 2.19. Proteins from the cells were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. A protein of approximately 14.5 kilodaltons (kD) was synthesized by cells containing pTHTH11A at higher levels in the presence of IPTG. A second protein approximately 18.0 kD in size was synthesized by pTHTH11B-containing cells at increased levels in the presence of IPTG (Fig. 19). Although they were stable in E. coli, recombinant plasmids isolated using this vector
Figure 19. Polyacrylamide gel of proteins from *E. coli* strains containing pMMB24 recombinant plasmids, stained with Coomassie Brilliant Blue as described in section 2.19. Protein samples electrophoresed were from strain HB101 containing the following plasmids: 1 and 2, no plasmid; 3 and 4, pTHTH11A; 5 and 6, pTHTH11B. Lanes 2, 4, and 6 contain proteins from cells grown in the presence of 5 mM IPTG. Arrows (▼) indicate proteins whose synthesis is detectably induced by IPTG. Molecular size standards are indicated in kD at the right.
The amp region appears to encode a 60 kD protein, synthesized by cells containing pH8205 (1425 inserts in fragments m1, n1 and n2), pH8685 (24 inserts in fragments m1 and Tn3-Hollo) insertion plasmids pH8566, pH8222, pH8686 and pH8687 (see Fig. 6 for subcloned fragments and Fig. 10 for transposon insertion positions). Other groups have demonstrated production of a protein of similar size from clones containing the amp region in m. Using the miniM13 technique (J. Schröder, personal communication).
underwent deletions in both the vector and insert when introduced into *Agrobacterium*, making this vector unsuitable for analysis of acc protein expression in the homologous host. Plasmid-encoded protein synthesis induced by IPTG was difficult to observe in Coomassie-stained acrylamide gels, as this stain is not very sensitive, and Coomassie-stained chromosomally-encoded proteins presented an unacceptably high background.

Using the more powerful maxicell technique, subcloned restriction fragments containing all or a portion of the acc region were analyzed for encoded protein products in *E. coli* strain LCD44. Plasmids pTHH206 and pTHSB5 (Fig. 6) encoded a protein of 60 kD (Fig. 20, lanes 4 and 7) that was not seen in strains containing only the vectors (Fig. 20, lanes 3 and 5). A protein of 48 kD was synthesized in cells containing pTHSE26 (Fig. 20, lane 2). No insert-specific proteins were detected in strains containing pTHSE16 or pTHSH11 (Fig. 20, lanes 8 and 9). Maxicell analysis of strains harboring the cloned acc region carrying different Tn3-HoHo1 insertions showed that the 60 kD protein was synthesized by all strains except those containing insertions 476 and 426 (see Fig. 10 for map positions) within EcoRI fragment 26 (Fig. 21, lanes 2 and 3). The 48 kD protein was not detectably synthesized by any of these strains.

The acc region appears to encode a 60 kD protein, synthesized by cells containing pTHH206 (HindIII fragments 31a, 11 and 38), pTHSB5 (BamHI fragment 5) and Tn3-HoHo1 insertion plasmids pTHB566, pTHB422, pTHB555 and pTHB587 (see Fig. 6 for subcloned fragments and Fig. 10 for transposon insertion positions). Other groups have demonstrated synthesis of a protein of similar size from clones containing the acc region in *E. coli* using the minicell technique (J. Schröder, personal communication, 91).
Figure 20. Maxicell analysis of subcloned fragments of the acc region. Subclones and vectors, indicated at the top of the figure, were introduced into *E. coli* strain LCD44 to visualize acc protein products. See section 2.19 for maxicell procedure and electrophoresis conditions. Shown is an autoradiogram of an acrylamide gel in which $^{35}$S-labeled proteins synthesized by maxicells have been separated. Molecular masses of protein size standards (lane 1) are shown in kD at the left. Arrows (▼) indicate insert-encoded polypeptides. Maxicell strain LCD44 contained: 2, pTHSE26 (pSa152::EcoRI 26); 3, pSa152 (vector); 4, pTHH206 (pSa4ΔH::HindIII 31α, 11, 38); 5, pSa4ΔH (vector); 6, no plasmid; 7, pTHSB5 (pSa152::BamHI 5); 8, pTHSE16 pSa152::(EcoRI 16); 9, pTHSH11 (pSa152::HindIII 11). See Fig. 6 for subcloned fragment positions.
Figure 21. Maxicell analysis of constructs containing Tn3-HoHo1 insertions into the ace region, in strain LCD44. Lanes 1 and 9, protein size standards, masses in kD given to the right of the figure; lanes 2 through 8 were loaded with equal amounts of radioactivity from cells containing plasmids: 2, pTHB476 (see Fig. 10 for transposon map positions); 3, pTHB426; 4, pTHB566; 5, pTHB422; 6, pTHB555; 7, pTHB587; 8, pCP13 (vector). Arrow (▶) indicates insert-encoded protein.
The image shows an SDS-PAGE gel with molecular weight markers (kD) ranging from 200 to 14.3. The gel contains nine lanes (1 to 9) with different protein patterns. The molecular weight markers are 200, 97.4, 68, 60, 43, 29, 18.4, and 14.3 kD. The gel is used to analyze the protein content and size of the samples.
Cells harboring vectors pSa4ΔH, pSa152, pCP13, plasmids pTHE16 (EcoRI fragment 16), pTHSE26 (EcoRI fragment 26), pTHSH11 (HindIII fragment 11), or pTHB476 or pTHB426, carrying insertions within EcoRI fragment 26, do not detectably synthesize this protein. Plasmid pTHSE26 instead encodes a 48 kD protein, and plasmids pTHTH11A and pTHTH11B (HindIII fragment 11 cloned in front of the tac promoter) appear to encode proteins of 14.5 and 18.0 kD, respectively.

In addition to analysis of acc-encoded proteins using maxicells, we attempted to demonstrate synthesis of polypeptides by this region using a commercial in vitro prokaryotic DNA-directed transcription-translation kit (Amersham). Plasmids pTHH206, pTHSE26, pTHVH11 and the vectors used in their construction, pSa4ΔH, pSa152, and pVK101 were assayed according to the instructions of the manufacturer. Equivalent amounts of incorporated radioactivity for each sample were subjected to SDS polyacrylamide gel electrophoresis, fluorography and autoradiography as described in section 2.19. In no case were insert-encoded proteins observed, indicating that the technique as it was performed was not helpful in studying acc-encoded proteins.


Strain C58 is not the only Agrobacterium strain known to be sensitive to agrocin 84, or to induce cancerous plant growths containing agrocinopines (50, 130, 133, 138, 196). Other isolates fitting one or both of these criteria include Agrobacterium tumefaciens strains T37, J73, K827, and A281, A. radiobacter strain K299, and A. rhizogenes strains A4 and 15834. Each of these strains was tested for its agrocin 84 reaction and the effect of agrocinopines on that reaction. Strains NT1 or C58C1RS
containing plasmids from these isolates were also analyzed (Table 7). Consistent with previous findings (50), virulent nopaline strains C58, T37 and K827 were sensitive to agrocin 84. Sensitivity was inducible in each case by agrocinopines A+B (Table 7). Agrocinopines also induce Ti plasmid conjugal transfer in these three strains (47). Tumorigenic nopaline strain J73 from South Africa and strain NT1 harboring its Ti plasmid displayed this same agrocin 84 reaction (Table 7). Sensitivity of the nontumorigenic nopaline A. radiobacter strain, K299, to agrocin 84 was also inducible by agrocinopines A+B. Both agropine-type A. tumefaciens transconjugant strain A281 and NT1 containing the nopaline catabolic plasmid pAtK84b of A. radiobacter strain K84 were resistant to the antibiotic (Table 7). Agrocin 84 sensitivity was not induced by agrocinopines A+B in either strain. Agrocinopine A does, however, induce conjugal transfer of pAtK84b (47). It has been reported that sensitivity to agrocin 84 in strain A281 could be induced by agrocinopines C and D (50), a finding which we confirmed (Table 7). Agrocinopines C and D are also the conjugal opines for pTiBo542 (47). Agrobacterium rhizogenes strains 15834 and A4 both displayed agrocin 84 sensitivity inducible by agrocinopines A+B. Susceptibility was encoded on pArA4a, rather than on the virulence plasmid, pRiA4, of the latter strain (Table 7). Uptake of agrocin 84 and agrocinopines A+B was not tested in most of these strains (see below).

Sensitivity to agrocin 84 that is agrocinopine-inducible, and agrocinopine-inducible conjugal transfer imply uptake of both opine and antibiotic. This is frequently the case in strains where both processes have been examined (50, 51, 133). Strains that transport and catabolize
Table 7. Opine-inducible agrocin 84 sensitivity of Agrobacterium strains containing various Ti, At, Ri, and Ar plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Agrocin 84 reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>acc inducer opine</th>
</tr>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>A. tumefaciens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>T37</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>J73</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>NT1(pTiJ73)</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>K827</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>K299</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>NT1(pAtK84b)</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>A281</td>
<td>R*</td>
<td>Agrocinopines C/D</td>
</tr>
<tr>
<td>C58C1RS(pArA4a)</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>C58C1RS(pR1A4)</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>A. rhizogenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
<tr>
<td>15834</td>
<td>S</td>
<td>Agrocinopines A/B</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by the plate bioassay. S, agrocin 84-sensitive; R, agrocin 84-resistant; R*, agrocin 84-sensitive only in the presence of inducer opines.

<sup>b</sup> - , no known inducer opine.

<sup>c</sup> - , acc absent.
the same compounds are likely to have similar transport systems, similarities which should be detectable at the DNA sequence level. To determine whether sequence similarities exist between the acc regions of these strains, DNA hybridization analyses with pTHH206 as a probe were used to identify regions of homology between the acc region of pTiC58 and plasmids purified from other Agrobacterium strains. Plasmid DNA from A. tumefaciens strains containing the classical nopaline Ti plasmids pTiK27 and pTiT37, as well as plasmid DNA from nopaline strain J73, all displayed restriction enzyme digestion patterns considerably different from the digestion pattern of plasmid DNA from strain C58 (Fig. 22 A and C, lanes 8-11). Despite these differences in restriction pattern, all three plasmid DNAs displayed strong hybridization patterns indistinguishable from those of pTiC58 when probed with pTHH206 (Fig. 22 B and D, lanes 8-11). The only other plasmid showing detectable acc sequence similarity was pAtK299, the opine catabolic plasmid from A. radiobacter nopaline strain K299, (Fig. 22 B and D, lane 7). However, the pattern of hybridizing restriction fragments from strain K299 plasmid DNA was unlike the patterns displayed by the DNAs of the virulent nopaline strains. Likewise, the total restriction pattern of strain K299 plasmid DNA in ethidium bromide-stained agarose gels differed greatly from the fragment patterns of plasmid DNA from strains T37, K827, J73, and C58 (Fig. 22 A and C, lane 7). No similarities in DNA sequence were observed between acc of pTiC58 and pAtK84b of strain K84 (B and D, lane 4). There was also no significant similarity detected between pTHH206 and pTiBo542 (B and D, lane 3), pArA4a (lane 6), and A. rhizogenes strain 15834 total plasmid DNA (lane 5).
Figure 22. 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 **BamHI** (A and B) or **EcoRI** (C and D, following page), subjected to agarose gel electrophoresis, and stained with ethidium bromide (A and C) as described in section 2.20. The DNA fragments were transferred to Genescreen™ filters and probed with 32P-labeled pTHH206 mixed with **HindIII**-cleaved lambda DNA. Autoradiograms of the filters are shown in B and D. 1, λ/HindIII; 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.
3.15. **Comparison of pTiC58 and pAtK84b ace regions.**

Plasmid pAtK84b enables *A. radiobacter* strain K84 to catabolize the opines produced by nopaline-type tumors. In addition, agrocinopines A+B induce conjugal transfer of both pTiC58 in strain C58 and pAtK84b in strain K84 (47). pAtK84b does not, however, show any sequence similarity to the pTiC58 ace region (Fig. 22), and does not confer sensitivity to agrocin 84 (Table 7). These results led us to ask whether pAtK84b confers uptake of agrocinopines and agrocin 84. To test this, strains C58 and NT1(pAtK84b) were incubated over a 48 h period at 28°C in small cultures containing 3 mM agrocinopines A+B. Paper electrophoresis of culture samples showed that both removed the opines from the culture medium (Fig. 23A). However, agrocin 84 uptake experiments performed as described in Chapter II showed that agrocin 84-sensitive strain C58 (Fig. 3) removed the antibiotic from culture media, while agrocin 84-resistant strain NT1(pAtK84b) (Table 7) did not transport agrocin 84 (Fig. 23B).
Figure 23. Agrocinopine and agrocin 84 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 hour timepoints indicated at the bottom of the figure and subjected to HVPE and alkaline silver nitrate staining. HVPE conditions and abbreviations are as described in the legend of Fig. 7. B. Strains C58, NT1(pTiC58Tra'), NT1, and NT1(pAtK84b) were tested for agrocin 84 transport as described in the legend of Fig. 15. Supernatants spotted in the center of the plates are from cultures of: 1, C58; 2, K438; 3, NT1; 4, NT1(pAtK84b). Incubation in hours is shown at the right.
4.1. One Transport System for Two Compounds.

Previous work has mapped the region responsible for sensitivity to agrocin 84 to the 8 o’clock sector of the pTiC58 map as it is conventionally depicted (Fig. 2 this work, refs. 83, 176). The studies of Ellis and Murphy (50) suggest that the true substrates for the system encoded in this region are the phosphorylated sugar opines, agrocinopines A and B. Using A. tumefaciens nopaline strain K57A, containing pTiK27, they showed that agrocinopine A induces agrocin 84 transport, and that the presence of agrocinopine A blocks agrocin 84 uptake. They also showed that mutant strains resistant to agrocin 84 fail to take up both the antibiotic and agrocinopine A. These data suggest the hypothesis that a single permease is responsible for the transport of both agrocin 84 and agrocinopines A and B. To further investigate this hypothesis, we have cloned and subcloned the acc region of pTiC58. We have created a series of transposon insertions into the region and have analyzed them for the phenotypes they confer. The data presented here strengthen the hypothesis. In all of the A. tumefaciens strains examined which contain cloned fragments of the pTiC58 acc region and in subsequent insertion mutants, sensitivity to agrocin 84 is in every case coincident with transport of both the antibiotic and the opines (Figs. 3, 6, 7, 8, 10, 11, and 16, Table 3). No clone or mutation has been found that gives only one or two of these phenotypes. The various clones confer a somewhat higher level of sensitivity on strain NT1 than does wild-type pTiC58. This may
be due to a dosage effect since vectors such as pCP13 and pVK102 are derived from RK2, a plasmid existing at 5 to 8 copies per cell (45). The Ti plasmid exists at a single copy per cell (62).

4.2. Effect of acc on Agrocin 84.

In uptake studies using radioactively labeled agrocin 84, Ellis et al. (51) found that cells containing pTiC58Tra' incubated with the labeled antibiotic first rapidly accumulate radioactivity to a maximum level of accumulated counts per min in five min, and then by 60 min lose a portion of it. To investigate whether this is due to Ti plasmid-mediated agrocin 84 breakdown, they introduced pTiC58Tra' into the same cell as the agrocinogenic plasmid pAgK84. Unlike strains containing only pAgK84, this strain, containing both pAgK84 and pTiC58Tra', does not secrete detectable agrocin 84 as determined by a plate bioassay. Wild-type pTiC58 in trans to pAgK84 has no such effect. Analysis of Tra' and agrocin-resistant mutants shows this lack of agrocin secretion to be associated with the presence of a constitutively-expressed acc region. One explanation Ellis et al. put forward was that the agrocin 84 is being taken up by the constitutively-expressed Ti plasmid-encoded permease as soon as it is secreted. Alternatively, they proposed that acc mediates breakdown of the antibiotic within the cell. The agrocin 84 uptake experiments of Ellis et al. (51) described above suggest that a constitutive acc region may mediate agrocin 84 degradation while a repressed region does not. We have shown that acc clones reduce the amount of detectable agrocin secreted from cells also containing a pAgK84::Tn5 derivative (Fig. 5). The secreted-agrocin-reduction phenotype associated with these active, multicopy cosmid clones is intermediate between those of pAgK84-containing
cells harboring the wild-type and transfer-constitutive Ti plasmids, paralleling the agrocin 84 sensitivity phenotype. This is consistent with the proposal that the level of acc expression influences the amount of agrocin 84 that accumulates in the medium. Whether this influence takes the form of agrocin 84 breakdown, transport of the antibiotic back into the cell, or both remains uncertain.

Ellis et al. (51) suggested the possibility that this proposed transport of agrocin 84 back into the cell would result in feedback inhibition of antibiotic biosynthesis. Evidence against inhibition at the expressional level was presented by Ryder et al. (147). Using RNA dot blot hybridizations and a probe containing the agrocin 84 biosynthetic region, they showed no detectable differences in hybridization levels to RNA from strains C58 or K439 containing pAgK84. This indicates that a constitutive acc region has no detectable effect on expression of agrocin 84 biosynthesis at the transcriptional level.

4.3. The Size of acc.

Since cosmid clones pTHB112, pTHH32, and subclone pTHH206 all encode agrocin 84 sensitivity, and transport of agrocin 84 and agrocinopines A + B (Figs. 3, 6, 7 and 8, Table 3), while subcloned fragments HindIII 11 and BamHI 5 are inactive (Agr'), HindIII fragments 31a and 11 must encode the entire regulon (Fig. 6). Agrocin 84 sensitivity conferred by pTHH206 is properly regulated by agrocinopines (Fig. 6), indicating that this clone contains the agrocinopine-agrocin 84 region and its regulatory gene.

We have mapped the agrocinopine-agrocin 84 region of pTiC58 to a segment of DNA from 5.3 to 6.9 kb in length; all Tn3-HoHol and Tn5 insertions tested within this DNA segment abolish agrocin 84 sensitivity,
and, when tested, agrocin 84 and agrocinopine uptake (Figs. 10, 11, 14, and 16). Analysis of phenotypes conferred by cosmid clones and subclones indicates that the left boundary of acc lies between the left ends of fragments HindIII 31a and EcoRI 16. The right limit is bounded by the right ends of fragments BamHI 5 and HindIII 11 (Figs. 4 and 6). Transposon mutagenesis has allowed a much more accurate definition of these limits. pTHH313 contains the nearest Tn5 insertion on the left side of acc not abolishing agrocin 84 sensitivity, positioning the left border of the regulon in the left half of HindIII fragment 31a, between the Tn5 insertions in pTHH313 and pTHH352 (Fig. 14). This is consistent with the results of Cooksey (30) who mapped an indigenous insertion sequence element in a spontaneous agrocin 84-resistant mutant of strain C58 to the middle of HindIII fragment 31a. It is also consistent with mapping results generated with transposons Tn5 (163) and Tn7 (83). pTHB587 contains the nearest Tn3-HoHoI insertion on the right side not affecting acc function, placing the right border of the region between this Tn3-HoHoI insertion and the right end of the cloned pTiC58 DNA in pTHB55 (Agr', Acp', see box in Fig. 10).

4.4. Does BamHI 17 Encode Agrocin 84 Sensitivity?

It has been reported that pTiC58 BamHI fragment 17, cloned in pBR322, encodes agrocin 84 sensitivity (163). Our results suggest that BamHI fragment 17 contains only a portion of one of the complementation groups (accE) and probably encodes only part of the transport system. Since BamHI fragment 17 lacks the left end of its complementation group, presumably including the transcript promoter, for the fragment to express, the vector must supply a promoter. It is possible that an agrocin 84
sensitivity function encoded on fragment 17 is expressed from the tetracycline resistance gene promoter present on pBR322. This would predict expressional polarity, with the fragment conferring sensitivity in one insertional orientation but not in the other. Sorman did not report on any such polar effect. Our clones, with BamHI 17 inserted into pBR322 in both orientations, fail to confer sensitivity to the antibiotic (Fig. 6). Although our strategy for stabilizing such recombinant clones in Agrobacterium was different from that used by Sormann, we do not believe this should make any difference. However, these workers used an agrocin 84 bioassay involving replica-plating colonies onto plates containing the antibiotic. Unlike the overlay technique, this assay can give highly variable results (33).

4.5. acc Contains Five Complementation Groups.

The complementation experiments performed with subclones and transposon insertions shed light on the number of genes encoded within acc. The region can apparently be divided into at least five complementation groups (Figs. 14A and 24). Each group may correspond to a single transcript or even to a single gene. Tn5 insertion 313 does not lie within acc, since this mutation does not abolish sensitivity to agrocin 84 (Fig. 14B). Complementation group A of acc spans Tn5 insertions 352, 301 and 349. These three mutations complement all Tn3-HoHol insertions except 476 and 426. Furthermore, these two Tn3-HoHol insertions within this group are complemented by BamHI fragment 5 but not EcoRI 26 (Fig. 13). The failure of the latter fragment to complement these two mutations indicates that part of accA lies to the right of EcoRI 26. HindIII 11 overlaps Tn3-HoHol insertion 426, but does not complement
it. This fragment does complement insertions 566 and 422, which are also complemented by fragments BamHI 5 and EcoRI 16 (Fig. 13). The HindIII 11 complementation pattern puts mutations 566 and 422 into complementation groups different than that of 476 and 426. The data for the SmaI 9 subclone, which complements mutations 476 and 426, but not 566, support the conclusion that insertions 476 and 426 lie in different complementation groups than 566 and 422 (Fig. 13). However, Tn3-HoHol mutations 566 and 422 behave differently with respect to Tn5 insertions 312, 306, and 304. Tn5 mutation 312 complements all Tn3-HoHol insertion mutations except 566, putting these two mutations into the second complementation group, accB. Likewise, since Tn3-HoHol insertion 422 is the only mutation not rescued by either Tn5 insertion 306 or 304, these three mutations constitute the third complementation group, accC. Tn5 insertions 348 and 314, both of which abolish agrocin 84 sensitivity, each complement all five Tn3-HoHol mutations, indicating that they lie within a fourth complementation group, accD. Lastly, Tn5 insertion 317 fails to rescue only Tn3-HoHol mutation 555, putting these two transposon insertions into the fifth group, accE (Fig. 14). Tn3-HoHol insertion 555 within this group is complemented by EcoRI 16 and HindIII 11, but not by BamHI 5 (Fig. 13). This is presumably because fragments EcoRI 16 and HindIII 11, which contain sequences to the right of BamHI 5, encode all the information for this complementation group while BamHI 5 does not. Fragments BamHI 5 and HindIII 11 together contain all the sequences necessary for acc function and reconstitute the region when combined in trans (Fig. 13A). Fragments EcoRI 16 and BamHI 5 do not complement each other, presumably because sequences to the right of EcoRI fragment 16 are
required for agrocin 84 sensitivity (Fig. 13A). Fragment EcoRI 16 overlaps Tn3-HoHol insertion 555 and Tn5 insertion 317, and complements the former (Figs. 13A and 14A). This fragment therefore contains all of accE. Yet, EcoRI 16 and BamHI 5 do not complement each other (Fig. 13A), indicating that sequences to the right of EcoRI 16 are required for agrocin 84 sensitivity. This suggests a sixth complementation group to the right of accE (Fig. 24).

4.6. Agrocinopine Sensitivity.

A surprising result of these complementation experiments is that certain merodiploid strains are sensitive not only to agrocin 84, but also to agrocinopines A+B (Fig. 13B). It may be that the opines are taken up but incompletely catabolized by these strains, so that a metabolic intermediate accumulates to toxic levels. This has been found in E. coli glycolysis mutants (89). An alternate explanation is that these merodiploids parallel the maltose-sensitive E. coli mutants containing lamB::lacZ fusions constructed by Silhavy et al. (157). They proposed that when certain LamB-LacZ fusion proteins are synthesized in large amounts, as when induced by maltose, they would jam the export machinery inserting them into the cell membrane, a lethal event. It may be that in certain of the merodiploid Agrobacterium strains containing acc::lacZ fusions and acc subclones, agrocinopine-induced transport proteins which have been truncated or fused to LacZ are also jamming the protein export machinery and killing the cells.

4.7. Transcriptional Direction of acc.

Only Tn3-HoHol insertions 476, 426, 422, and 555, transcriptionally oriented from left to right (Fig. 10) or in a clockwise direction on the
Figure 24. The \textit{acc} region. Restriction map showing the complementation groups of the \textit{acc} region. Transcriptional direction, when indicated by analysis of Tn3-HoHol insertions, is indicated by arrows. Solid lines bounded by vertical bars indicate the minimum size of each group. Dashed lines indicate uncertainty of maximum complementation group size. The functions or probable functions associated with the groups, as determined in or as suggested by this work, are indicated below the map.
BamHI

HindIII

EcoRI

Detectable proteins

60 kD (periplasmic binding protein?)

48 kD (truncated 60 kD?)

Agrocin 84 Sensitivity\(^a\)

- + + + + + + +?

Agrocinopine transport

N.T. + + + N.T. + N.T.

Agrocin 84 Transport\(^b\)

N.T. + + + N.T. + N.T.

Regulation

negative regulator

positive regulator?

\(^a\) -, not required; +?, probably required.

\(^b\) N.T., not tested; +, required.
pTiC58 map of Depicker et al. (41), showed β-galactosidase activity. This indicates that accA, accC, and accE are transcribed from left to right as shown in Fig. 24, and is consistent with the presence of a coordinately regulated region read in a clockwise direction on the Ti plasmid.

4.8. Expression of acc in E. coli.

Evidence suggests that transcription of the region is active in both A. tumefaciens and E. coli, yet agrocin 84 sensitivity and agrocinopine transport are not expressed in E. coli (Table 5, Figs. 3B and 7B). It is known that some Ti plasmid genes are transcribed and translated in E. coli (78, 165, 200). It has recently been shown that although mannityl opine catabolic determinants from an octopine Ti plasmid do not allow growth of E. coli on the appropriate opine, mannopine cyclase activity (42) can be detected in cell-free extracts from such strains (43). However, this activity in E. coli is low and is not inducible by its substrate as it is in Agrobacterium. This lack of induction by the proper opine suggests that the compound may not enter the cell. Our observation that E. coli harboring pTHB112 fails to take up the sugar phosphate opine (Fig. 7B) is certainly consistent with this interpretation. On the other hand, in their study of the octopine catabolism region (occ) of octopine-type plasmid pTiA6, Stachel et al. (165) observed low levels of octopine-inducible β-galactosidase activity in E. coli cells containing occ::lacZ fusion plasmids. These plasmids did not confer octopine utilization on E. coli. However, opine utilization was scored by assessing growth, not uptake, so it may be that octopine can enter E. coli cells and induce occ, but is not further metabolizable.
4.9. **Multiple Transcripts Within ace**.

Were *ace* composed of a single transcript with only a single promoter, each transposon insertion should exert polar effects on all genes downstream, and no Tn5 insertion should have complemented any Tn3-HoHol mutant. That complementation is observed suggests two possibilities. Either the region consists of a single transcript, and genes downstream of each insertion are being expressed from promoters on the transposons, or there are additional promoters within the region. Expression of genes adjacent to Tn5 insertions from Tn5-encoded promoters, independent of transposon orientation, has been reported (11), but this expression is usually weak, and is only found in a fraction of the insertion mutants. We have shown that most Tn5 and Tn3-HoHol insertions within the *ace* region are able to complement each other in trans, generating agrocin 84-sensitive phenotypes (Fig. 14). The conclusion that best fits these data is that multiple transcripts are present. An alternative possibility is that *ace* is transcribed as a single unit, but contains contiguous internal promotors that function as well. In being composed of several independently expressed genes, *ace* appears to be unique; it differs from the arabinose regulon (153) in that apparently all *ace* functions are closely genetically linked. It also differs from most periplasmic transport systems, such as those for arabinose (80), branched-chain amino acids (112), histidine (76), oligopeptides (77), ribose (9), maltose (75), and phosphate (171, see 4 for review), which are or appear to be operons transcribed as single units.

The *ace* regulon parallels these systems, though, in several ways. *ace* appears to encode a periplasmic binding protein, a common feature of
these systems. Transport functions for most of the systems indicated above, including ace, are encoded by multiple, coregulated genes, with a regulatory function encoded at one end of the region. In such systems, the transport functions are mediated by two or more components. There are genes for one or two (in the cases of histidine and branched-chain amino acid permeases) periplasmic binding proteins, three cell membrane-bound components, and additional genes involved in catabolism (ribose operon, ref. 9) or of unknown function. Considering the observations that single insertions throughout the region abolish ace function, including opine uptake; that in pairs, these insertion mutations define five complementation groups; and that there is a Ti plasmid-encoded agrocin 84-binding periplasmic protein fraction (133), the ace regulon appears to encode a multicomponent transport system similar to those that have been studied.

4.10. ace Allows Agrocinopine Catabolism.

Experiments demonstrating growth on agrocinopine A by agrobacteria containing a nopaline type Ti plasmid have been reported (50), but the experiments were not quantitative, and the appropriate negative controls were absent. Analysis of growth on agrocinopine B shows that ace, contained on pTHH206, allows utilization of this opine as a sole source of carbon and energy (Fig. 8). The studies presented here show a marked increase in cell number associated with uptake of agrocinopine B only for cells containing the ace region. Strain NT1 containing pTHB112 (Agr' Acp') does reach a higher cell density than NT1 containing pTHH206 (Agr' Acp'). This may be due to plasmid copy number differences between pTHB112 and pTHH206 (45, 55), or to possible additional growth advantages conferred
by the former plasmid. The noncatabolizing strain NTl fails to take up the opine and shows no growth over the 72 h culture period (Fig. 8). Agrocinopines are therefore not only conjugal opines, but are also capable of serving as a sole source of carbon and energy.

These results indicate that agrocinopines A and B, rather than the toxic agrocin 84, are the legitimate substrates for this transport system (50). Of the three designations used for the region, we favor acc (125) for agrocinopine catabolism over psc (152) for phosphorylated sugar catabolism or ags (125) for agrocin sensitivity. This is more consistent with designations of other opine catabolic determinants including occ (octopine catabolism, 63) and noc (nopaline catabolism, 83).


Experiments were performed to detect products of agrocinopine catabolism using cell-free extracts of an acc-constitutive strain mixed with agrocinopine B. Using HVPE in a borate buffer, we wanted to demonstrate metabolic intermediates with mobilities differing from the substrate. These could then be characterized in part using different buffer systems and staining techniques. In one such experiment, a cell-free extract of strain K439 apparently converted the opine to a faster-migrating silver nitrate-positive compound (Fig. 9). The result is weakened, however, because the experiment was not repeatable. Despite attempts using toluenization instead of sonication (see sections 2.18 and 3.7), and descending paper chromatography instead of HVPE (see sections 2.12 and 3.7), the result could not be repeated. It may be that the growth phase at which the cells are harvested is critical and must be precisely measured. The sonication regime used may not have been optimal;
others of longer and shorter duration might be tried. Use of staining techniques other than alkaline silver nitrate may avoid the abundant, obscuring spots observed on descending paper chromatograms. There may exist membrane-bound components involved in agrocinopine catabolism, and toluenization of the cells could have disrupted their function.

A protocol for the synthesis of agrocinopines lacking chemical blocking groups has recently been published (115). Chemical synthesis of the opine should eliminate the problem of limited agrocinopine availability associated with much of this work and allow use of larger, more readily visible amounts of substrate in catabolism experiments. Alternatively, radioactively-labeled opine could be used.

4.12. Transcriptional Regulation of acc.

Results presented in Fig. 12B indicate that agrocinopines A+B transcriptionally regulate the agrocinopine-agrocin 84 region of pTiC58. The opines cause increases in the β-galactosidase activity of strains containing lacZ fusions at either end of acc, again consistent with a coordinately regulated region. That Tn3-HoHo1 insertions within the agrocinopine-agrocin 84 region are associated with uninduced β-galactosidase activities four- to 30-fold above enzyme levels in strains lacking Tn3-HoHo1 or carrying a Tn3-HoHo1 insertion outside the region (Fig. 12A and B) is not unexpected. Strain C58 is sensitive to agrocin 84 in the absence of the opine inducer, suggesting that the uninduced agrocinopine-agrocin 84 region is transcribed at a relatively high basal level. Strain C58 has a detectable basal expression of acc that is inducible, while strains K439 and NT1(pTiC58Tra') represent the fully constitutive state. This contrasts with strains containing the agropine-
type Ti plasmid pTiBo542. These strains show complete resistance to agrocin 84, but become sensitive to the antibiotic in the presence of its inducer opine, agrocinopine C (50).

The Tn3-HoHol insertion derivatives are not inducible for β-galactosidase activity in the strain NTL background, presumably because these transposon insertion plasmids do not encode all the necessary components of the agrocinopine transport system (Fig. 12A). Lack of a functional permease in these strains would explain failure to transport the sugar opines (Fig. 11), a necessary prerequisite for induction of acc. Clear induction by agrocinopines, seen when the Tn3-HoHol derivatives are assayed in strain K439 (Fig. 12B), supports this hypothesis. The Ti plasmid in this strain presumably supplies the agrocinopine transport system allowing uptake of the opine and consequent induction of the lacZ fusion on the coresident recombinant element.


Subcloned pTiC58 fragment EcoRI 26 negatively affects agrocin 84 sensitivity (Table 4), agrocin 84 uptake (Fig. 17A), agrocinopine uptake (Fig. 17B), acc transcription (Table 5), and Ti plasmid conjugal transfer (Table 6) when supplied in trans to acc. For those phenotypes that have been tested, this is also true for HindIII fragment 31a, which is entirely contained within EcoRI fragment 26 (Table 4, Fig. 17). These are the results one would expect for the trans-acting negative regulatory element proposed by Ellis et al. (51) for acc and tra, and by Petit et al. (142) and by Klapwijk et al. (107, see 101 for review) for acc and tra.

EcoRI fragment 26 present in trans has a strong negative effect on the acc phenotypes of strains containing wild-type pTiC58 or pTiC58Tra†
(Fig. 17, Tables 4 and 5). This is probably attributable to a smaller number of repressor molecules synthesized by the single-copy Ti plasmid (62) compared to the multicopy subclone pTHSE26 (55). In a strain containing pTiC58 alone, there is a single repressor locus synthesizing regulator molecules that act on a single acc region. The addition of a multicopy subclone carrying only the repressor locus would presumably raise the number of repressor molecules acting on the one acc region several fold. The complete abolition of agrocin 84 sensitivity and transport may seem difficult to reconcile with the approximately 30% reduction in acc transcriptional activity associated with the presence of pTHSE26 in trans to acc::lacZ fusions (Table 5). However, it is important to remember that the acc region acted upon in this case, an acc::lacZ fusion, is carried on a vector that exists in multiple copies (45). Many copies of a repressor synthesized by multiple copies of its gene will probably have a stronger effect on a single copy target, the Ti plasmid, than on a target region such as the Tn3-HoHol-induced acc::lacZ fusion plasmids, existing at several copies per cell.

Further evidence that EcoRI fragment 26 encodes the tra-acc regulator is provided by experiments using subcloned fragments of pTiC58Tra<sup>c</sup>. If this plasmid is mutated in a locus encoding a negative regulatory element for tra and acc, a restriction fragment containing the locus should have a reduced negative effect or no negative effect at all on either tra or acc when supplied in trans. Fragment EcoRI 26 from this Tra<sup>c</sup> plasmid, when introduced in trans to either the entire Tra<sup>c</sup> Ti plasmid, or wild-type pTiC58, has no detectable repressive effect on agrocin 84 sensitivity (Table 4). This pTiC58Tra<sup>c</sup> fragment in trans to
pTiC58Tra⁶ also has no detectable negative effect on conjugal transfer (S. Beck Von Bodman, personal communication). In contrast, the same EcoRI 26 restriction fragment from the wild-type Ti plasmid, cloned into the same vector in the same orientation (S. Beck Von Bodman, personal communication), has strong negative effects on both agrocin 84 sensitivity and conjugal transfer (Tables 4 and 6). This difference also holds true for the larger BamHI 5 fragment, which contains EcoRI 26. The wild-type BamHI 5 fragment, cloned in pSal52, has a regulating effect on the agrocin 84-supersensitive phenotype associated with pTiC58Tra⁶. Agrocin 84 sensitivity of strain UIA143(pTiC58Tra⁶, pTHSB5) is inducible by agrocinopines (Table 4). The same BamHI fragment cloned from the Tra⁶ Ti plasmid in the same vector has no repressive effect on agrocin 84 sensitivity encoded by either the wild-type or the Tra⁶ Ti plasmid (Table 4). These results all support the hypothesis that pTiC58Tra⁶ carries a mutation in the tra-acc regulator. The fragment to which we have assigned this function in the wild-type case has no negative effects on acc or tra if it is isolated from the Tra⁶ Ti plasmid. One can conclude from this that pTiC58Tra⁶ contains a mutation in fragment EcoRI 26, that this mutation is indeed probably within a common regulatory gene for tra and acc, and that it is at least in part responsible for the constitutive tra and acc phenotypes of strains harboring this Ti plasmid.

Interestingly, an Agrobacterium merodiploid containing cosmid clone pTHH7, which contains HindIII fragment 31a and sequences to its left (Fig. 4), together with a Tra⁶ Ti plasmid, is sensitive to agrocin 84 at a markedly reduced level (Table 4). This phenotype is also approximated by strains containing recombinant plasmids with Tn3-HoHo1 insertions in acc
in trans to a Tra\(^c\) Ti plasmid (Table 4). However, when pTHB55 is the cosmid clone placed in trans to pTiC58Tra\(^c\) (Fig. 4), the strain is now sensitive to agrocin 84 at the wild-type level, induced to a higher level of sensitivity by agrocinopines A+B (Table 4). pTHB55 contains BamHI fragment 5 (within which smaller EcoRI fragment 26 is positioned left of center) and sequences to the left of BamHI 5. Similarly, Agrobacterium strains containing either subcloned fragment HindIII 31a or EcoRI 26 in trans to pTiC58Tra\(^c\) are resistant to agrocin 84. Placing the BamHI fragment 5 in trans to the Tra\(^c\) Ti plasmid results in a wild-type level of agrocin 84 sensitivity, inducible by agrocinopines. An increase in agrocin 84 sensitivity is seen when sequences to the left and right of EcoRI 26 are added in trans to the pTiC58Tra\(^c\), as with the EcoRI 26 and BamHI 5 subclones, and cosmids pTHH7 and pTHB55. This might be explained by repressor binding sites present in the sequences flanking EcoRI 26, so that the repressor is titrated out. A candidate for such a site has recently been located. Beck Von Bodman et al. (8) have isolated Tn5 insertions in pTiC58 that confer a Tra\(^c\) phenotype. These mutations, mapping to the EcoRI fragment 33 which lies to the left of the EcoRI fragment 26 (Fig. 4), are not complemented to a repressed or regulated Tra phenotype by overlapping wild-type cosmid clones and their subclones, including subcloned EcoRI 33. Since EcoRI fragment 33 does not exert a negative effect in trans, it probably does not encode a diffusible negative regulatory function. Instead, a cis-acting site encoded by this fragment is suggested. EcoRI fragment 33 did derepress wild-type strain C58 for conjugal transfer, consistent with the hypothesis that this fragment is titrating the repressor. However, the possibility remains
that EcoRI 33 may encode a diffusible positive regulatory function. The physiological consequences of elevated and unbalanced acc and tra protein expression in the merodiploid strains may contribute to these unusual phenotypes as well.

It is also conceivable that some function that counteracts repression, possibly an activator, is encoded within BamHI 5 fragment to the right of EcoRI 26. Sormann (163) has postulated that an activator of conjugal transfer is located in precisely this region. This conclusion was based on studies of a Tn5 insertion into fragment HindIII 11 in a wild-type acc clone containing HindIII fragments 3, 3la, and 11. When the transposon in this clone was homogenotized into pTiC58Tra+, the plasmid no longer showed constitutive transfer. Sormann proposed that the insertion interrupted a function that stimulates conjugal transfer. An alternate explanation, however, is that the presumably defective regulator gene of the Tra+ plasmid was replaced with the functional wild-type one, encoded on EcoRI 26, during the recombination event. The tra genes would now be regulated, effectively Tra+, unless agrocinopines were present. Sormann did not report experiments to determine if this homogenote could be induced for conjugal transfer by agrocinopines A and B.


The map positions and functions or possible functions of the five defined acc complementation groups are shown in Fig. 24. Two, possibly three activities have been identified in the acc region: the presumably multicomponent agrocinopine-agrocin 84 transport system, and a repressor controlling both acc and tra expression. If the acc-tra and the occ-tra regions are similarly organized, it is probable that acc also encodes an
agrocinopine catabolic activity. In the \textit{occ-tra} system, octopine transport and catabolic functions are encoded by \textit{occ} (106, 107). It is also possible, however, that once agrocinopines are taken into the cell by the \textit{acc} transport system, they are broken down by chromosomally-encoded enzymes. From the structure of agrocinopine B, it is conceivable that a single function, a phosphodiesterase for example, could produce readily catabolized metabolic intermediates. The agrocinopine B catabolism experiment discussed above (section 4.11) may have detected this activity in constitutive strain K439 (Fig. 9).

\textit{Tn}5 insertion 313 within fragment \textit{EcoRI} 26, which defines \textit{accR}, has apparently inserted into the \textit{acc-tra} repressor (Figs. 14 and 24). pTHH313 confers agrocin 84 sensitivity that is not further inducible by agrocinopines. However, the opines appear to interfere slightly with agrocin 84 sensitivity in strain UIA143 harboring this plasmid (Fig. 14B). Furthermore, the mutation caused by insertion 313 does not generate an agrocin 84-supersensitive phenotype. This is different from the phenotype of strains containing pTiC58\textit{Tra}c, which is thought also to have a mutated repressor function. The mutation in pTiC58\textit{Tra}c results in an agrocin 84-supersensitive phenotype. This is the predicted phenotype that a mutation abolishing the \textit{tra-acc} repressor would have. Agrocinopines have no effect on this agrocin 84-supersensitive phenotype (Fig. 3) unless they are supplied in greater concentration, where they interfere slightly with agrocin 84 sensitivity (S. Beck Von Bodman, personal communication). Interference by agrocinopines with agrocin 84 sensitivity is therefore observed both with insertion mutant 313, and with the \textit{Tra}c mutant at higher opine levels. This may be due to competition between opine and antibiotic
for the transport system, as suggested by Ellis and Murphy (50). The difference between the two agrocin 84 sensitivity phenotypes associated with pTHH313 and pTiC58TraC may be due to the differing natures of the mutations causing them. The simplest explanation for the noninducible, nonconstitutive phenotype associated with insertion 313 is that it alters, rather than inactivates, the repressor, so that agrocinopines no longer have an effect on repression. The insertion may instead affect both the repressor and another function to generate its peculiar phenotype. Alternatively, the physiological effects of elevated gene expression by the subclone may again be playing a role in the phenotype. Complementation group A, next to accR (Fig. 24), cannot encode the repressor, since homogenotization of Tn3-HoHol insertion 476 into pTiC58 does not result in a derepressed transfer phenotype (Table 6).

One of the five acc complementation groups may encode the identified opine catabolic activity (Fig. 8) and another could encode a component of the previously-identified agrocin 84-binding periplasmic protein fraction (Fig. 24 this work, ref. 133). The possibility that there are separate periplasmic binding proteins encoded within acc for agrocin 84 and the agrocinopines cannot formally be ruled out. However, this seems unlikely; Kerr and Tate (105) have pointed out that suicide genes have no biological (or evolutionary) advantage. Ellis and Murphy (50) have provided additional evidence for a single binding protein by showing that agrocinopines will block agrocin 84 uptake when the opine is present in excess molar concentrations. The simplest explanation for these data is that the two molecules are competing for the same periplasmic binding protein.
Complementation groups A through E presumably all encode functions required for sensitivity to agrocin 84 (Fig. 24). Each and every one of the five Tn\(^3\)-HoHol and nine Tn5 transposon insertions into the five complementation groups abolishes agrocin 84 sensitivity (Figs. 10 and 14). Groups A, B, C, and E presumably all encode essential components of the agrocinopine/agrocin 84 transport system, since Tn\(^3\)-HoHol insertions into these four regions abolish transport of both agrocin 84 and agrocinopines (Figs. 11, 16, and 24). Complementation group D is defined solely by Tn5 insertion mutants, which were tested only for agrocin 84 sensitivity, and not for opine or antibiotic uptake. A conclusion therefore cannot be made concerning the requirement of accD for agrocin 84 or agrocinopine transport. If one of the complementation groups also encodes an agrocinopine catabolic activity, acc may encode a multifunctional protein involved in both transport and catabolism. This has been reported for other periplasmic transport systems, such as the histidine permease and the branched-chain amino acid permease (110, 112).

If the region contained a single transcript, all transposon insertions should have polar effects on all functions downstream, and insertions mapping upstream should not complement mutations mapping downstream. Instead, all nine of the Tn5 insertion mutants tested are complementable by at least three of the five Tn\(^3\)-HoHol mutations analyzed (Fig. 14A). In 35 out of 45 cases, one mutation complements a second mutation mapping to its right, indicating that polarity of insertional mutations within acc is not an important factor.

The information gained about the complementation groups of acc can be used to explain previously isolated mutants. Ellis et al. (51)
isolated a Tra \(^{e}\) mutant of pTiC58 called pWI1003, discussed in section 1.9. pWI1003 no longer confers agrocin 84 sensitivity. This phenotype could be explained by a small deletion between the regulatory gene, encoded by acc\(R\), and acc\(A\) that abolishes both functions. Restriction digestions of Ti plasmid DNA from this mutant revealed one or more deletions within fragments EcoRI 26, HindIII 31a, and BamHI 5, consistent with this hypothesis (S. Beck Von Bodman, personal communication).

4.15. The Protein Products of acc.

Maxicell analysis showed that *E. coli* strains containing acc clones pTHH206, pTHB112, or pTHSB5 synthesize a 60 kD protein (Fig. 20 and 21). Proteins of a similar size encoded by this region have been reported by two other groups (J. Schröder, personal communication, 91). The 60 kD protein is not seen in maxicells containing insertion plasmids pTHB476 and pTHB426, indicating that the gene encoding this protein spans these two insertions. Thus the protein is encoded by acc\(A\) (Fig. 24).

The acc\(A\)-encoded 60 kD protein is a good candidate for a periplasmic binding protein (Fig. 24). It is similar in size to other such proteins (75), and, as determined by maxicells (Figs. 20 and 21), appears to be produced in abundant quantities, as are other periplasmic binding proteins (4, 34, 75). A high affinity periplasmic transport system for glucose has also been recently described for *A. radiobacter* that consists of at least three components (34). Two of the components are glucose binding proteins synthesized at high concentrations under glucose-limited conditions. Finally, the acc\(A\) product is encoded at the left end of a region transcribed from left to right. This is also the case for a number of
periplasmic transport systems, such as histidine (76), ribose (9), and phosphate (4, 171).

A second protein of 48 kD was observed in maxicells containing the subcloned EcoRI 26 fragment (Fig. 20). This 48 kD protein may be unique, and a possible candidate for the tra-acc co-regulator. However, recalling that EcoRI 26 lies at the left end of a region apparently transcribed from left to right, the 48 kD protein is more likely a truncated form of the 60 kD polypeptide (Fig. 24). The following observations support this interpretation. The 60 kD and 48 kD proteins are synthesized in approximately equal amounts, and they are never seen together in one strain. The 48 kD protein is not synthesized by cells containing either the subcloned BamHI fragment 5, which contains EcoRI 26; pTHH206, which contains all of acc; or any of the Tn3-HoHol insertion plasmids examined (Figs. 20 and 21). Finally, the DNA sequence between the 476 insertion point and the right border of EcoRI fragment 26 could readily accommodate such a 48 kD truncated protein.

The regulatory function is therefore probably encoded in the region of EcoRI fragment 26 to the left of insertion 476, within accR (Fig. 24). Evidence to support this is provided by plasmid pTHH313, whose Tn5 insertion maps to the left of Tn3-HoHol insertion 476 (Fig. 14A). As discussed in section 4.14, this plasmid confers agrocin 84 sensitivity with an altered inducibility phenotype (Fig. 14B), suggesting that the Tn5 insertion lies within a gene encoding the regulatory function.

An IPTG-inducible protein of approximately 14.5 kD is synthesized by E. coli cells containing HindIII fragment 11 cloned downstream of the tac promoter of pMMB24 (Fig. 19). This protein may be a truncated,
carboxy-terminal fragment of the 60 kD protein encoded within accA, or it may be encoded within one of the complementation groups to the right (Fig. 24). There may be an open reading frame present in the opposite direction on fragment HindIII 11 which encodes the 18.0 kD protein seen in pTHTH11B-containing cells only in the presence of IPTG (Fig. 19). This is inconsistent with the transcriptional direction of acc as determined by Tn3-HoHol insertions, and this is therefore probably not a protein or part of a protein synthesized by the wild-type acc region.

It is possible that no other proteins were seen because they are synthesized at levels too low to detect with the methods used. This is true of membrane-bound components of other bacterial periplasmic transport systems, including histidine (5), ribose (87), and phosphate (4, 171).

4.16. acc-homologous Regions of Other Agrobacterium Strains.

The pTiC58 acc region is highly similar to regions on the nopaline/agrocinopine-type Ti plasmids pTiT37 and pTiK27 (Fig. 22). An identical hybridization pattern for plasmid DNA of the agrocin-producing nopaline strain J73 suggests that this Ti plasmid contains a region closely related to the acc region of classical nopaline strains. Ethidium bromide-stained restriction patterns of these plasmids differ considerably (Fig. 22A and C), suggesting that either only segments of these plasmids are closely related, or that they contain many RFLPs. Plasmid pAtK299 of nopaline-type A. radiobacter strain K299 is the only other element tested showing notable sequence similarity to the acc region. The hybridizing fragment pattern for plasmid pAtK299 is markedly different from the hybridization patterns of classical nopaline plasmids, indicating a more
distant evolutionary relationship between this nontumorigenic plasmid ace region and the nopaline-type tumorigenic plasmid ace regions analyzed.

Plasmids from *A. rhizogenes* strains show little or no similarity to ace (Fig. 22B and D), indicating that regions different enough from each other to lack detectable DNA sequence similarity can still encode sensitivity to the same antibiotic. Agrocin 84 sensitivity in *A. rhizogenes* strains is inducible by agrocinopines A+B, the region responsible for this phenotype being located on the avirulent Ar plasmid (Table 7). Agrocinopine A has been found in hairy roots induced by strain 15834 (138), consistent with the idea that the *A. rhizogenes* Ar plasmid region encodes agrocinopine catabolism as well as agrocin 84 sensitivity. The hybridization studies indicate that even regions encoding catabolism of other phosphorylated sugar opines can be different; pTiBo542, which presumably encodes catabolism of agrocinopines C and D, shows no detectable sequence similarity to the agrocinopine A and B catabolic region (Fig. 22). Agrocinopines C and D are, however, structurally different from their A and B counterparts (M. Ryder, personal communication), and agrobacteria containing pTiBo542 are only sensitive to agrocin 84 when induced with agrocinopines C and D (50).

Most interesting is the lack of sequence similarity between the ace region of pTiC58 and its counterpart on pAtK84b of strain K84 (Fig. 22). Conjugal transfer of both plasmids is inducible by agrocinopines A and B (47), the two plasmids display considerable sequence similarity to each other (124), and they belong to the same incompatibility group (Farrand, Hong and Slota, submitted for publication). While both plasmids confer agrocinopine transport (Fig. 23A), only the pTiC58 ace regulon encodes
antibiotic transport and sensitivity (Figs. 3 and 23B, Table 7). The difference is a logical one; it would be disadvantageous for strain K84, which contains both the agrocinogenic plasmid pAgK84 and the opine catabolic plasmid pAtK84b, to be sensitive to the antibiotic it produces. Plasmid pAgK84 of this strain confers immunity to agrocin 84, so that an altered acc region on pAtK84b that no longer transports agrocin 84 may seem superfluous. However, it may be that the immunity function evolved after agrocin biosynthesis, so that an alteration in acc was necessary. Alternatively, pAtK84b may have acquired its agrocin 84-resistant trait while harbored in another strain unprotected by immunity functions, and was subsequently introduced into what is now known as strain K84.

Based on Southern hybridization, agrocin 84 sensitivity and agrocinopine uptake analyses, there appear to be four classes of acc regions (Table 8). The first includes the prototype, pTiC58. *Agrobacterium tumefaciens* plasmids pTiC58, pTiT37, and pTiJ73 show identical patterns of hybridization to the subcloned pTiC58 acc region (Fig. 22). pAtK299 of *A. radiobacter* strain K299 also shows sequence similarity to the pTiC58 acc region, but displays a different pattern of hybridizing DNA fragments, indicating the presence of RFLPs (Fig. 22). All four of these plasmids confer agrocin 84 sensitivity inducible by agrocinopines A+B (Table 7). As was argued in section 3.9, concerning Fig. 12, uptake of the opine is necessary for induction of acc expression. Therefore all four of these plasmids probably confer agrocinopine uptake. This has been confirmed for pTiC58 (Fig. 7 this work, ref. 50), pTiK27, and pTiT37 (50). The second class contains only one member of the plasmids studied, pArA4a of strain *A. rhizogenes* strain A4. This plasmid
Table 8. Classes of acc regions.

<table>
<thead>
<tr>
<th>Class</th>
<th>Source</th>
<th>Agrocinopine Transport</th>
<th>Agrocin 84 Sensitivity</th>
<th>Sequence Similarity to acc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pTiC58</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Prototype</td>
</tr>
<tr>
<td></td>
<td>pTiT37</td>
<td>+</td>
<td>S</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>pTiK27</td>
<td>+</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pTiJ73</td>
<td>N.D.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>S</td>
<td>+</td>
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<td></td>
<td>pAtK299</td>
<td>N.D.</td>
<td>S</td>
<td>+&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>II</td>
<td>pArA4a</td>
<td>N.D.</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>pTiBo542</td>
<td>-</td>
<td>S*&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>pAtK84b</td>
<td>+</td>
<td>R</td>
<td>-</td>
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</table>

<sup>a</sup> +, plasmid confers agrocinopine transport; -, opine transport not conferred.

<sup>b</sup> N.D., Not determined.

<sup>c</sup> S, agrocin 84-sensitive; R, agrocin 84 resistant; agrocin 84 sensitivity was inducible in each case by agrocinopines A+B.

<sup>d</sup> S*, agrocin 84-sensitive only in the presence of agrocinopines C or D.

<sup>e</sup> +, strong hybridization to cloned acc probe; -, little or no detectable hybridization to probe.

<sup>f</sup> Homology displayed RFLPs.
also confers agrocin 84 sensitivity inducible by agrocinopines A+B (Table 7). pArA4a shows no detectable sequence similarity with the acc region of pTiC58 (Fig. 22). The third class of acc regions contains pTiBo542 of A. tumefaciens strain Bo542 and transconjugant strain A281. No DNA sequence similarity is displayed with the acc region of pTiC58, pTiBo542 does not confer agrocinopine uptake, and agrocin 84 sensitivity is only conferred in the presence of agrocinopines C or D (50). The fourth class is represented by pAtK84b of A. radiobacter strain K84. It displays no detectable sequence similarity with the pTiC58 acc region (Fig. 22) and does not confer agrocin 84 sensitivity or transport (Table 7, Fig. 23B). However, this plasmid does encode agrocinopine transport functions (Fig. 23A).

The work included in this dissertation shows that the acc region of A. tumefaciens strain C58, and presumably other classical nopaline-type strains, is complex, consisting of at least five genes, spanning over five kb of DNA, that are probably separately transcribed (Fig. 24). The arguments presented by Ames (4) to justify the complexity of transport operons in E. coli and Salmonella typhimurium are valid for acc as well. A multicomponent mechanism may be necessary to transport substrate against a concentration gradient as the opine concentration increases within the cell. The high efficiency of these types of transport systems may require a complex structure. Such complexity is also not surprising if one considers the acc region in relation to the way of life of Agrobacterium. If the opine concept is correct, the goal of this organism is to create an ecological niche for itself. Its unique nutritional source, the opines synthesized in the plant tumor it induces, also serves to stimulate the
transfer of its tumorigenic principle, the Ti plasmid, to avirulent agrobacteria. This increases the efficiency with which the niche is utilized. The newly virulent organisms can incite more tumors, increasing the amount of opines synthesized. This transfer probably considerably enhances the virulence capacity of the genus, and allows more agrobacteria access to an increased opine supply. Without the acc region, the transport system for this conjugal stimulus and nutritional source, members of the genus Agrobacterium would probably be less efficient colonizers of the niche they engineer.
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APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

26 November 1989  [Signature]
Date  Director's Signature