Organization and Expression of the Genes on pAgK84 That Encode Production of Agrocin 84

Chang-Lin Wang,[1] Stephen K. Farrand,[1,2] and Ingyu Hwang[1]

[1]Department of Plant Pathology and [2]Department of Microbiology, University of Illinois at Urbana/Champaign, Urbana 61801 U.S.A.

Received 16 December 1993. Accepted 14 April 1994.

Agrocin 84 biosynthesis genes located in a 21-kb segment of pAgK84 were characterized by mutagenesis with Tn*3*HoHo1 and complementation analysis. Three overlapping fragments of the 21-kb segment, cloned into pRK415 or pLAFR6, were mutagenized with Tn*3*HoHo1, and 94 independent insertions were mapped and oriented. A series of merodiploid strains, each containing a Tn*5* insertion in pAgK84 affecting agrocin 84 biosynthesis, and a clone containing a Tn*3*HoHo1 insertion that blocks antibiotic production in homogenotes were constructed to determine the number of complementation groups involved in agrocin 84 biosynthesis. Five complementation groups were identified and named *agnA* through *agnE*. Analysis of *lacZ* fusions formed by the Tn*3*HoHo1 inserts indicated that all of the loci except *agnD* are transcribed in an anticlockwise direction. Insertions carried on clones and insertions marker-exchanged into pAgK84 had similar patterns of expression. The five *agn* loci were not expressed at significant levels in *Escherichia coli* DH5α grown in minimal or rich media. Levels of expression in *Agrobacterium tumefaciens* NT1 differed for each region; *agnA* was expressed at relatively high levels, *agnC* and *agnE* at intermediate levels, and *agnB* and *agnD* at very low levels. Similar patterns of expression were observed in minimal media, regardless of the carbon source, and at neutral and acidic pHs. Expression levels were lower in cells grown in rich medium. The level of expression of each *agn* locus was not affected by the presence of other *agn* genes or by the presence or absence of the large nopaline plasmid pAtK84b, present in strain K84. Nor were the levels of expression influenced by the addition of opines or root exudates to the culture media. All five *agn* loci were expressed at all growth stages, and expression reached maximum levels during late exponential growth. The *agn* loci were expressed *in planta,* and the patterns of expression were similar to those seen in bacteria grown *in vitro*. The presence of pAtK84b did not affect *agn* expression *in planta*.

Additional keywords: agn genes, biocontrol.

Crown gall, a tumorous disease of dicotyledonous plants, is caused by the gram-negative soil bacterium *Agrobacterium tumefaciens*. The pathogen infects susceptible plants at wound sites or root lenticels. During the infection, a portion of a large virulence plasmid present in the bacterium, the Ti plasmid, is excised and transferred to the host plant. This transferred DNA (T-DNA) becomes integrated into the plant cell genome. Enzymes encoded by genes on the integrated T-DNA catalyze the overproduction of auxin and cytokinins by the transformed cells, leading to hyperplastic proliferation. The uncontrolled cellular proliferation produces the characteristic crown gall tumors. Many of the details of this process are now understood and are the subject of recent reviews (Winans 1992; Zambryski 1992).

Crown gall disease is of economic importance to stone fruit and nut orchardists and to nurseries involved in the propagation of fruit tree stocks and ornamentals such as chrysanthemums and roses (Alconero 1980; Kennedy and Alcorn 1980). Because the tumors characteristic of the disease result from the genetic transformation of plant cells, crown gall can be controlled only by blocking events that occur prior to the integration of the T-DNA into the plant cell genome. In this regard, an effective biocontrol agent is available to protect plants from crown gall disease. The protective agent, an avirulent isolate of *A. radiobacter* called strain K84, is used commercially worldwide (Moore and Warren 1979).

The effectiveness of strain K84 as a biological control agent is related, in part, to the production by this organism of a highly specific antiagrobacterial antibiotic called agrocin 84. Disease caused by virulent agrobacteria susceptible to the antibiotic generally can be controlled, while that caused by pathogens innately resistant to agrocin 84 usually is thought to be refractory to protection by strain K84. Susceptibility of virulent agrobacteria to agrocin 84 is a trait conferred by certain, but not all, types of Ti plasmids, which accounts for the high degree of specificity shown by the antibiotic (Moore and Warren 1979).

Agrocin 84 is a disubstituted fraudulent analog of adenine nucleotide (Tate *et al.* 1979). It contains 3′-deoxy-D-arabinose in place of ribose and thus lacks a 3′ OH group. The antibiotic is believed to act as a DNA replication inhibitor by causing premature chain termination (Murphy *et al.* 1981). A glucofuranoside substituent at the N^6 position probably is re-

Present address of C.-L. Wang: Department of Bacteriology and Biochemistry, University of Idaho, Moscow, ID 83843 U.S.A.

Corresponding author: Stephen K. Farrand, Department of Plant Pathology, University of Illinois at Urbana/Champaign, N519 Turner Hall, 1102 S. Goodwin Avenue, Urbana, IL 61801 U.S.A.

MPMI Vol. 7, No. 4, 1994, pp. 472-481

quired for specific, high-affinity uptake of the antibiotic by a Ti plasmid–encoded metabolic system (Murphy *et al.* 1981). This system, encoded by *acc,* is responsible for the uptake and catabolism of agrocinopines A and B (Hayman and Farrand 1988), two unique sugar phosphodiester compounds. These are members of a class of metabolites called opines, which are found only in plant neoplasias induced by pathogenic agrobacteria (Dessaux *et al.* 1992). Hence, the antibiotic apparently mimics a natural tumor-produced metabolite that can be catabolized by the susceptible bacteria as a carbon and energy source. The function of the methyl pentanamide substituent at the C-5′ position is unknown. The structure of the active molecule suggests that the biosynthetic pathway may be complex.

Production of agrocin 84 by strain K84 is encoded by a 48-kb conjugal plasmid called pAgK84 (Ellis *et al.* 1979; Slota and Farrand 1982). Results from Tn*5* insertion mutagenesis and limited complementation analysis indicate that the genes encoding production of the antibiotic span about 21 kb of the plasmid and are organized into five complementation groups (Ryder *et al.* 1987). The plasmid also confers immunity to agrocin 84 in susceptible agrobacteria. Immunity is encoded independently by two regions of pAgK84, one mapping within complementation group I and the other within complementation group IV (Ryder *et al.* 1987). Recombinant clones encoding either region alone are sufficient to confer immunity. However, mutations on the intact plasmid mapping to one immunity region or the other abolish agrocin 84 production, but have no effect on immunity itself. This suggests that there are two mechanisms of plasmid-associated immunity and that both are related to biosynthesis of the antibiotic.

Nothing is known concerning the pathway for the biosynthesis of agrocin 84. Nor is it known if the expression of the genes encoding agrocin 84 biosynthesis is regulated. Production of agrocin 84 by strain K84 is generally assayed on minimal defined media. No zones of growth inhibition against susceptible indicator strains are evident when the assay is performed on rich media. However, it is not clear whether this is due to a repression of production by strain K84 or an inhibition of susceptibility to the antibiotic associated with growth of the indicator strain on rich medium. Finally, it is not known whether strain K84 produces the antibiotic *in situ,* a requirement if agrocin 84 is to play a role in the biocontrol process. In this work, we constructed a series of *lacZ* reporter fusions to genes, called *agn* genes, involved in agrocin 84 biosynthesis. These fusions were used to determine the organization of the genes in the *agn* region of pAgK84, to examine the patterns of expression of these genes at the transcriptional level, and to determine whether the genes are expressed when a bacterium harboring pAgK84 colonizes plant roots.

RESULTS

Subcloning and Tn*3*HoHo1 mutagenesis.

Previous studies located the genes encoding agrocin 84 biosynthesis on a contiguous 21-kb segment of pAgK84 (Farrand *et al.* 1985). Prior to mutagenesis with a reporter transposon, we subcloned this region as a set of three overlapping fragments. The 6.6-kb *Bam*HI C fragment, encoding complementation group I and part of group II, was cloned in both orientations into pRK415 to generate pBC4 and pBC5. The 16.5-kb *Pst*I fragment, overlapping complementation groups III, IV, and V, was cloned into pLAFR6 to produce pLA. An *Eco*RI partial clone, containing fragments G, J, and K, which overlap the inserts in pBC4, pBC5, and pLA, also was generated in pLAFR6. The insert in this clone, called pLE13, is about 5.3 kb in size and encodes complementation group II.

Each of these clones was separately mutagenized with Tn*3*HoHo1 as described in Materials and Methods. A total of

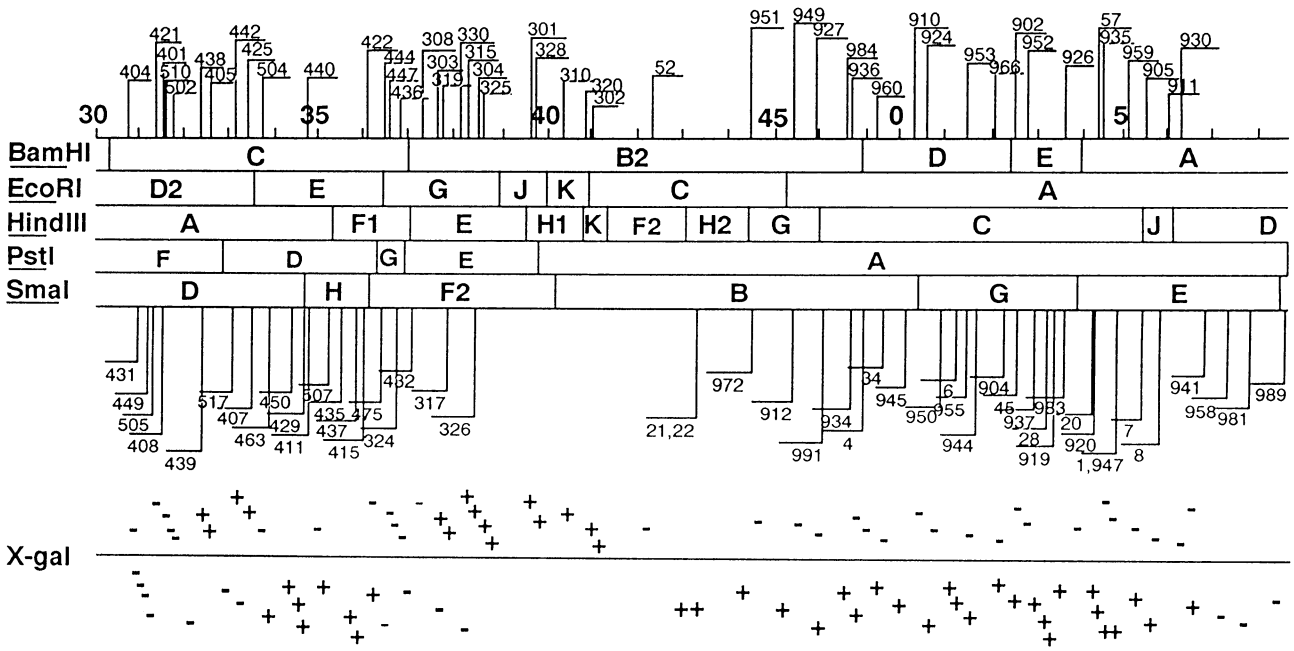

Fig. 1. Tn*3*HoHo1 insertion map of the 21-kb segment of pAgK84 involved in agrocin 84 biosynthesis. A total of 94 independent Tn*3*HoHo1 insertions were mapped as described in the text. Crossbars on each insertion indicate the transcriptional direction of the Tn*3*HoHo1 *lacZ* gene. β-Galactosidase activity, as determined on X-Gal plates, is indicated (+ or –) for each insertion.

94 independent insertions were isolated, mapped, and oriented (Fig. 1). The 400 and 500 series of insertions were produced in pBC4 and pBC5, respectively, while the 300 series was isolated in pLE13. The remaining insertions were produced in pLA. In *Escherichia coli* DH5α, all Tn*3*HoHo1 insertion derivatives of pLA and pLE13 produced white colonies when cultured on a medium containing X-Gal. Insertions in pBC4 and pBC5 produced blue, light blue, or white colonies. Blue colonies always were associated with orientations of the Tn*3*HoHo1 insertion which could be transcribed from the vector-encoded *lacZ* promoter. For this reason, we studied only the insertions in these two clones in which the *lacZ* fusions are oriented in the direction opposite that of the vector promoter. All such insertions gave rise to colonies of DH5α that were light blue or white on X-Gal plates.

Because of the relatively low level of expression in *E. coli,* each of the mapped insertion mutants was transferred into *A. tumefaciens* NT1, and the constructs were tested on medium supplemented with X-Gal. With the exception of insertions 958, 981, and 989, all of the fusions in pLA that are oriented from right to left (anticlockwise on pAgK84) yielded X-Gal$^+$ colonies, while all of those oriented in the opposite direction produced colonies that remained white on indicator plates (Fig. 1). In contrast, all insertions oriented from left to right in pLE13 (the 300 series; see Fig. 1) yielded X-Gal$^+$ colonies. The two insertions in the opposite orientation gave rise to white colonies on medium containing X-Gal. The orientations of X-Gal$^+$ insertions in pBC4 and pBC5 indicate that this fragment contains two transcriptional units expressed in opposite directions (Fig. 1). One set of X-Gal$^+$ insertions maps to the right half of *Bam*HI fragment C and is transcribed from right to left. The second active set maps to the left half of the fragment and is transcribed from left to right. Taken as a whole, the results suggest that the region covered by the three clones encodes a minimum of four transcriptional groups (Fig. 1).

Selected X-Gal$^-$ clones were introduced into NT1-(pAgK84) to determine if an activator activity encoded by the wild-type plasmid affects the expression of any of the fusions. Colonies of all such merodiploid strains remained white on X-Gal plates (data not shown).

Marker exchange mutagenesis.

Twenty-eight Tn*3*HoHo1 insertions, chosen to span the entire agrocin 84 biosynthesis region, were independently marker-exchanged into wild-type pAgK84. The positions of these insertions and a set of corresponding Tn*5* insertions previously mapped to this region (Farrand *et al.* 1985; Ryder *et al.* 1987) are shown in Figure 2. About half of the marker-exchanged Tn*3*HoHo1 insertions resulted in an Agn$^-$ phenotype. However, the three insertions mapping to the far right (8, 911, and 941) and the four insertions mapping to the far left of the region (401, 405, 442, and 425) had no detectable effect on agrocin 84 biosynthesis. Several insertions mapping in the interior of the 21-kb region also had no detectable effect on agrocin 84 production. These insertions correspond in their locations to a similar set of Agn$^+$ Tn*5* insertions, and they divide the *agn* locus into five regions (Fig. 2). Strains containing the marker-exchanged Tn*3*HoHo1 insertions showed patterns of β-galactosidase expression on X-Gal

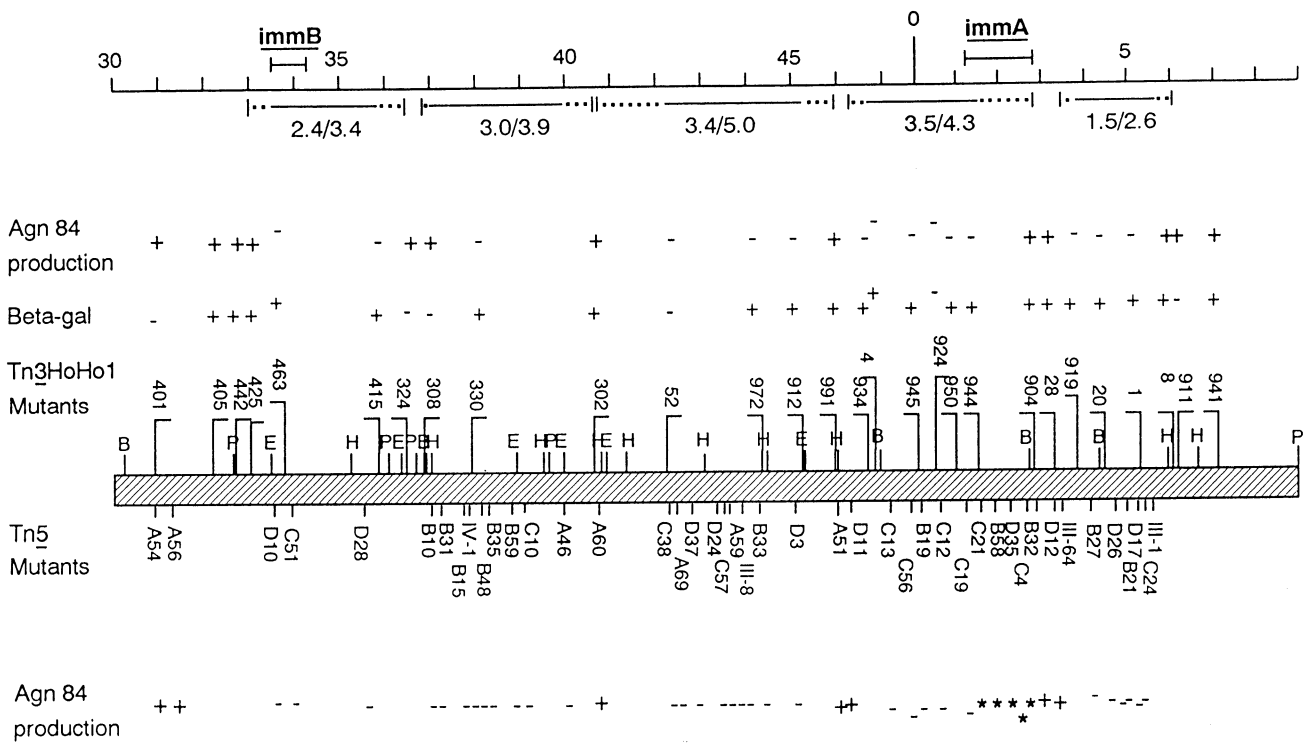

Fig. 2. Map positions of Tn*5* and Tn*3*HoHo1 insertions in the agrocin biosynthesis region of pAgK84. β-Galactosidase activity, as determined on X-Gal plates, is indicated (+ or −) for each Tn*3*HoHo1 insertion. Agrocin 84 production was assayed against *Agrobacterium tumefaciens* strains C58 and K439 as described in Materials and Methods. + = Agrocin 84 production ; − = no agrocin 84 detected; * = greatly reduced production of agrocin 84. The extent of each *agn* locus is indicated by solid and dotted lines below the coordinate (kb); the values given beneath these lines are minimum and maximum size estimates for each *agn* locus. *immA* and *immB* are the two immunity regions.

plates identical to those associated with the clones used to produce the homogenotes.

Complementation analysis.

A series of merodiploid strains were constructed to determine the number of complementation groups involved in agrocin 84 biosynthesis. Each harbored a derivative of pAgK84 containing a single Tn*5* insertion affecting agrocin 84 biosynthesis and a clone containing a Tn*3*HoHo1 insertion which, in homogenotes, also blocks antibiotic production. As a control for complementation, strains harboring each pAgK84::Tn*5* insertion derivative were transformed with the appropriate wild-type clone that overlapped the site of the insertion. All merodiploid strains were tested for restoration of agrocin 84 biosynthesis with the supersensitive indicator strain K439. As shown in Figure 3, restoration of agrocin 84 production was observed in some merodiploid strains, but not in others. Analysis of the results indicates that the biosynthetic region can be divided into five complementation groups, and that these correspond to the five regions separable by transposon mutagenesis. We propose the symbol *agn* to describe the genes required for agrocin 84 biosynthesis, and we have named the five complementation groups *agnA* through *agnE*. The extent of each locus and its direction of transcription are presented in Figure 3.

Expression of *agn* loci.

As determined by β-galactosidase levels from Tn*3*HoHo1-generated *lacZ* fusions, the five *agn* loci were not expressed at significant levels in *E. coli* DH5α grown in minimal or rich media (data not shown). Levels of expression in *A. tumefaciens* NT1 differed for each region; *agnA* was expressed at relatively high levels, while *agnC* and *agnE* were expressed at intermediate levels, and *agnB* and *agnD* were expressed at very low levels in all media tested (Table 1). Testing additional insertions in each locus yielded qualitatively similar results, although the absolute levels of β-galactosidase activity differed somewhat from one insertion to another in the same locus (data not shown). No significant β-galactosidase activity was detected from inserts oriented in the direction opposite that of the transcription direction of each *agn* gene (data not shown).

The level of expression of each *agn* locus was not affected by the presence of the other *agn* genes; β-galactosidase ac-

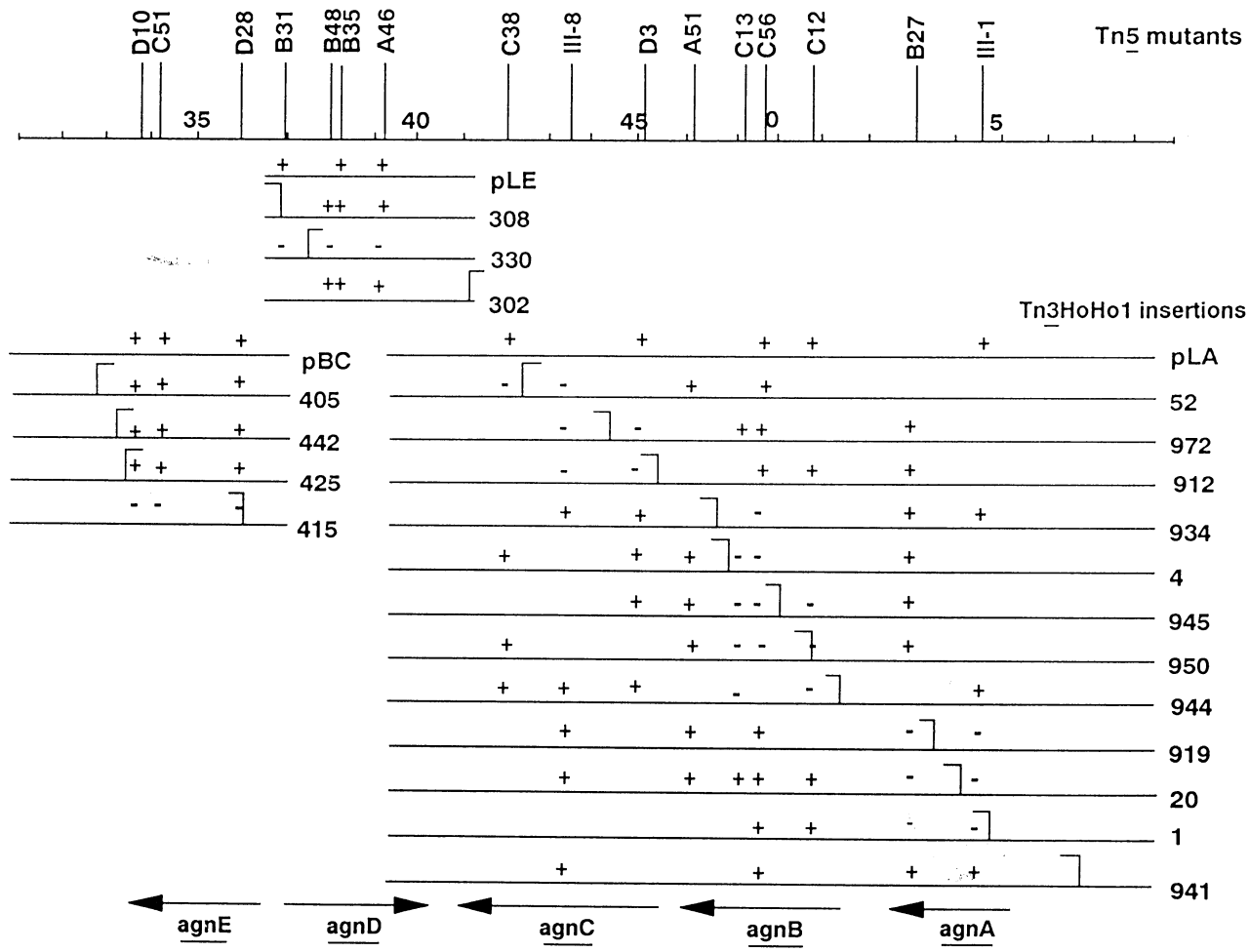

Fig. 3. Complementation analysis of the region encoding agrocin 84 biosynthesis. Recombinant plasmids containing Tn*3*HoHo1 insertions located in agrocin production regions were introduced into Tn*5* insertion derivatives of pAgK84 as described in the text. Each merodiploid was assayed for the production of agrocin 84 against *Agrobacterium tumefaciens* C58. + = Agrocin 84 production; – = no agrocin 84 detected. Five complementation groups were identified and named *agnA* through *agnE*. The arrows indicate the extent of each *agn* locus and the inferred direction of transcription.

Table 1. Expression of *agn::lacZ* fusions under various growth conditions

Growth conditions	β-Galactosidase activity per 10^9 cfu[a]				
	agnA	***agnB***	***agnC***	***agnD***	***agnE***
L broth	142.9	10.8	33.5	16.3	59.2
AB medium + carbon sources					
Mannitol	302.2	10.6	140.1	40.0	131.8
Sucrose	273.3	8.8	133.6	33.1	90.2
Glucose	250.4	5.5	132.7	36.5	125.0
Succinate	300.2	9.6	147.9	36.2	118.5
Arabinose	249.0	7.9	112.7	26.5	84.0
Stonier's minimal medium	286.5	14.6	182.1	35.9	111.1
vir "induction medium"	213.1	16.3	183.1	47.5	187.0
+ Nopaline (1 mM)	241.0	18.9	111.4	37.5	132.0
+ Agrocinopines A and B (25 μM)	237.6	13.8	151.1	24.6	81.0
AB medium + mannitol					
+ Nopaline (1 mM)[b]	214.9	14.6	130.5	28.6	127.7
+ Agrocinopines A and B (25 μM)[b]	277.6	13.2	128.1	26.4	104.3
+ Tobacco root exudate[b]	264.0	19.8	138.1	46.8	96.6

[a] Bacterial strains used for expression of *agn::lacZ* fusions were as follows: *agnA*, NT1(pAgK84::Tn*3*HoHo1-20); *agnB*, NT1(pAgK84::Tn*3*HoHo1-945); *agnC*, NT1(pAgK84::Tn*3*HoHo1-972); *agnD*, NT1(pAgK84::Tn*3*HoHo1-330); and *agnE*, NT1(pAgK84::Tn*3*HoHo1-415).
[b] Assayed in strain NT1(pAtK84b) containing the indicated *agn::lacZ* fusion plasmids.

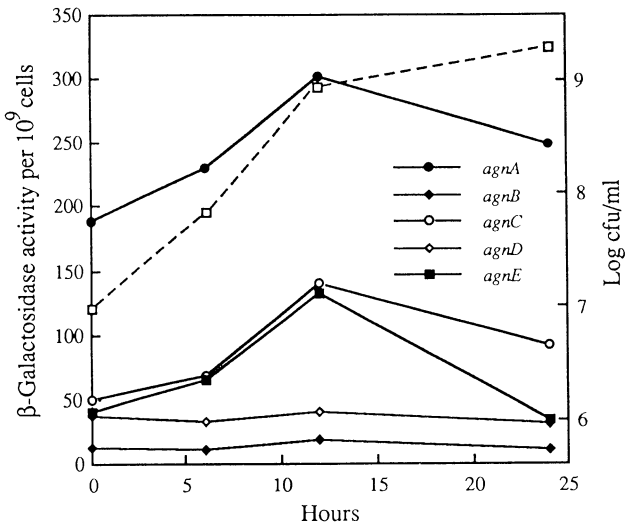

Fig. 4. Expression of *agn* loci during growth in AB minimal medium containing 0.2% mannitol. β-Galactosidase activity was measured as described in the text. Bacterial strains used for expression of *agn::lacZ* fusions were the following: *agnA*, NT1(pAgK84::Tn*3*HoHo1-20); *agnB*, NT1(pAgK84::Tn*3*HoHo1-945); *agnC*, NT1(pAgK84::Tn*3*HoHo1-972); *agnD*, NT1(pAgK84::Tn*3*HoHo1-330); and *agnE*, NT1(pAgK84::Tn*3*HoHo1-415). Growth kinetics and final yields of each test strain were very similar to each other. One such growth curve, representing all of the tested strains, is indicated by the dotted line.

tivities in strains containing the *lacZ* fusions in any of the clones were the same in the presence or absence of a wild-type copy of pAgK84 (data not shown). The presence of the large nopaline-catabolic plasmid pAtK84b did not influence the level of expression of any *agn* locus (data not shown).

Expression of each of the homogenotized reporter fusions was lowest when the cells were cultured in L broth (Table 1). While levels of β-galactosidase from the test fusions were higher in defined media, expression was not significantly different in cells grown in AB minimal medium containing glucose, succinate, arabinose, mannitol, or sucrose as the sole source of carbon (Table 1). Expression of each *agn* locus in cells grown in Stonier's minimal medium was similar to that

Table 2. Expression of *agn::lacZ* fusions *in planta*

agn::lacZ fusion[a]	Number of bacteria (10^7 cfu per root system)		β-Galactosidase activity per 10^9 cells	
	−pAtK84b	+pAtK84b	−pAtK84b	+pAtK84b
agnA	1.2	1.4	175.7	180.4
agnB	1.2	1.1	40.8	35.5
agnC	1.4	1.5	73.8	82.8
agnD	1.0	1.1	36.5	38.4
agnE	0.7	1.2	55.3	54.2
Control	1.5	NT[b]	17.0	NT

[a] Bacterial strains used for expression of *agn::lacZ* fusions were as follows: *agnA*, NT1(pAgK84::Tn*3*HoHo1-20); *agnB*, NT1(pAgK84::Tn*3*HoHo1-945); *agnC*, NT1(pAgK84::Tn*3*HoHo1-972); *agnD*, NT1(pAgK84::Tn*3*HoHo1-330); and *agnE*, NT1(pAgK84::Tn*3*HoHo1-415). Strain NT1 was used as a control.
[b] NT = not tested.

observed in AB minimal medium (Table 1). To determine if opines affect the expression of *agn* loci, nopaline or agrocinopines A and B were added to AB medium and to *vir* "induction medium." The expression of any of the five *agn* loci was not influenced by the addition of these opines to either medium (Table 1). Levels of β-galactosidase were not influenced by growth at pH 5.5 in a medium in which phosphate was limiting or by the addition of tobacco root exudate to AB minimal medium (Table 1).

All five *agn* loci were expressed at all growth stages. Expression reached maximum levels during the late exponential stage when the cells were grown in AB mannitol minimal medium (Fig. 4).

Expression of *agn* genes *in planta*.

β-Galactosidase activity from *lacZ* fusions of each *agn* locus was measured in bacterial cells colonizing tomato roots. Bacterial populations recovered from root systems 2 wk after inoculation as described in Materials and Methods ranged from 10^6 to 10^7 cfu per root system. Assays for β-galactosidase levels in the cells recovered from the roots indicated that all five *agn* loci were expressed *in planta* in patterns and at levels similar to those observed in cells grown in AB minimal

medium (Table 2). The numbers of bacterial cells recovered from tomato roots 2 wk after inoculation were similar among strains harboring different *agn* mutants. The presence of the nopaline-catabolic plasmid pAtK84b did not influence the degree of root colonization or the levels of *agn* expression *in planta* (Table 2).

DISCUSSION

By a combination of mutagenesis with Tn*3*HoHo1, homogenotization, and complementation analysis, we have determined the boundaries of and the gene organization within the region of pAgK84 required for the biosynthesis of agrocin 84. The *agn* region spans 19.6–20.6 kb of contiguous DNA. The leftmost (anticlockwise) boundary lies within 0.5 kb between the Agn⁻ Tn*3*HoHo1 insertion 463 at coordinate 33.5 and the Agn⁺ Tn*3*HoHo1 insertion 425 at coordinate 33 (Fig. 2). This is a considerably more precise definition of the left boundary than was earlier reported from analysis of Tn*5*-generated mutants (Farrand *et al.* 1985; Ryder *et al.* 1987). The rightmost (clockwise) boundary lies within 0.5 kb between the Agn⁺ Tn*3*HoHo1 insertion 8 at coordinate 5.9 and the Agn⁻ Tn*5* insertion III-1 at coordinate 5.4 (Fig. 2). It is unlikely that genes involved in the biosynthesis of agrocin 84 lie outside of this zone. Saturation mutagenesis with Tn*5* clearly showed that regions of pAgK84 outside of *agn* are not required for agrocin production (Farrand *et al.* 1985).

Results from complementation analyses (Fig. 3) and the observation that certain Tn*5* and Tn*3*HoHo1 insertions mapping within *agn* do not demonstrably affect the production of agrocin 84 indicate that the 20-kb biosynthetic region is organized into at least five transcriptional groups. These groups, named *agnA* through *agnE*, correspond to complementation groups V through I as defined by Ryder *et al.* (1987). Group *agnB* may be composed of two transcriptional units. Mutations in this region produce two phenotypic classes. Those to the right, including Tn*5* insertions C21, B58, D35, and C4, result in a greatly diminished, but still detectable level of agrocin 84 production (Fig. 2). Insertions mapping to the left half of *agnB* result in a completely Agn⁻ phenotype. Analysis of Tn*3*HoHo1-generated *lacZ* fusions in this region indicates that *agnB* is transcribed from right to left (Figs. 2 and 3; see below). If *agnB* is a single transcriptional unit, transposon insertions located to the right side should be polar on the downstream determinant, and their phenotypes should be Agn⁻. However, we cannot rule out the possibility that Tn*5* insertions may not be polar and may promote expression of a downstream gene. Tn*5*-mediated gene expression in *Agrobacterium* was reported previously (Piper *et al.* 1993). Since we were not able to separate the two by complementation analysis, we define this as a single group until more information is available. Interestingly, immunity region 2 (*immA*) maps to the right side of *agnB* (Figs. 2 and 5).

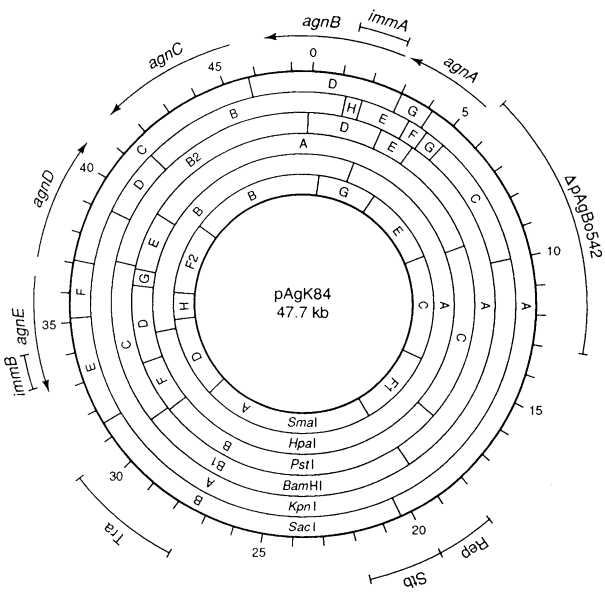

Fig. 5. Functional map of pAgK84. The agrocin 84 biosynthesis determinants *agnA* through *agnE* are defined as described in the text. The direction of transcription of each agn locus, as indicated by the arrows, were determined from the β-galactosidase activity resulting from each insertion. *immA* and *immB* are the two immunity regions. ΔpAgBo542 represents the portion of pAgK84 absent from the closely related agrocinogenic plasmid pAgBo542 (Slota and Farrand 1982). The locations of the conjugal transfer region (Tra) and the replication origin (Rep/Stb) were previously described (Farrand *et al.* 1985, 1992).

The second immunity function, *immB,* encoded by pAgK84, maps to *agnE* (complementation group I). Previous analysis indicates that this region encodes at least two functions separable by mutant phenotype (Ryder *et al.* 1987). Both are required for agrocin 84 biosynthesis, but only one is required for immunity. Thus, the 2.2-kb *agnE* locus most likely encodes at least two polypeptides. There is a fairly large intergenic region between *agnC* and *agnD* (Figs. 2 and 3). Only two insertions, Tn*5*-A60 and Tn*3*HoHo1-302, map to this approximately 2-kb region. However, neither of these insertions has any detectable effect on antibiotic production, which makes it unlikely that an undetected biosynthetic or regulatory locus resides in this region. Minimum and maximum size estimates for each group, based on a compilation of results from mutagenesis with the two transposons, are shown in Figure 2. These sizes suggest that *agn* could encode between seven and 12 polypeptides.

Reporter fusions between the *agn* determinants and *lacZ* show that four of the five groups are transcribed in an anticlockwise direction (Figs. 1, 2, and 5). All X-Gal⁺ Tn*3*HoHo1 insertions in *Pst*I fragment A of pLA are oriented in one direction. However, complementation analysis divided this region into three groups (Fig. 3) corresponding to *agnA*, *agnB,* and *agnC*. Only *agnA* is expressed at high levels under the culture conditions tested (Table 1). The other four transcriptional groups are expressed at levels two to 20 times lower than that observed for *agnA* (Table 1). Detectable expression of the *agn* genes is consistent with the fact that strains harboring pAgK84 produce agrocin 84 on a variety of minimal media. The fact that all *agn* genes were expressed through all growth stages is consistent with previous observations that agrocin 84 production is coincident with cell growth during the culture cycle (Formica 1990; Murphy 1982).

The *agn* genes are not expressed at significant levels in *E. coli,* regardless of the medium on which the strains are grown (data not shown). Some *Agrobacterium* genes are expressed well in *E. coli* (Stachel *et al.* 1985; Farrand *et al.* 1989). Others, such as some of those encoding opine catabolism (Dessaux *et al.* 1987) seem not to be expressed in enteric hosts.

The lack of expression of *agnA* in *E. coli* is a function of its promoter. A 550-bp fragment containing the *agnA* 5′ region expresses *lacZ* and *cat* fusions at high levels in *Agrobacterium, Rhizobium,* and *Pseudomonas* hosts, but gives very low levels of expression in *E. coli* (C.-L. Wang, S. Beck von Bodman, and S. K. Farrand, unpublished).

Expression of the *agn* genes does not appear to be strongly regulated. Although several of the loci are expressed at relatively low levels, this is evidently sufficient to provide enough agrocin 84 to kill susceptible *Agrobacterium* cells. β-Galactosidase levels were lowest in cells cultured in L broth (Table 1). Intermediate levels of expression were observed in cells grown in minimal salts media. The *agn* genes do not appear to be strongly regulated by carbon catabolite repression. Like other members of the Rhizobiaceae, *Agrobacterium* seems to prefer succinate over sugars as a carbon source (Nautiyal *et al.* 1992; Hong *et al.* 1993). However, levels of *agn* gene expression were as high in medium supplemented with succinate as they were in medium containing any of the other substrates tested. Factors such as low phosphate and low pH, which are known to affect expression of the Ti plasmid *vir* genes (Winans 1990), have no detectable effect on expression of the *agn* genes. Expression of *agn* genes in Stonier's minimal medium, which is used as a standard medium for assaying agrocin 84, was similar to that in AB minimal medium. Constitutive expression of *agn* genes differs from expression of genes required for oomycin A production by *Pseudomonas fluorescens* Hv37a. This antibiotic suppresses the development of disease caused by *Pythium ultimum* on cotton and other crops, and expression of genes required for oomycin A production is regulated by glucose (Gutterson *et al.* 1988).

Several studies have shown that *A. radiobacter* K84 colonizes tomato roots and that this colonization ability is important to the biocontrol process (Kerr and Htay 1974). Strain K84 contains the large nopaline-catabolic plasmid pAtK84b (Ellis *et al.* 1979). However, there are no indications that this plasmid plays any role in biocontrol. Our works show that the presence of pAtK84b did not affect *agn* gene expression or root colonization, indicating that the plasmid may not contribute to either of these phenotypes.

The observation that *agn* genes are expressed *in planta* is consistent with the hypothesis that the production of agrocin 84 by strain K84 in the rhizosphere is related to biocontrol of crown gall caused by susceptible agrobacteria. Similar correlations relating biocontrol to *in situ* production of antibiotics have been reported for pseudomonads that control *Pythium* diseases in cotton (Howie and Suslow 1991) and take-all of wheat (Thomashow *et al.* 1990). These systems appear to establish the importance of antibiosis as a mechanism involved in at least some biocontrol processes (Farrand 1991; Schroth and Hancock 1981). It should be noted that strain K84 can control crown gall caused by pathogens that are resistant to agrocin 84 (Cooksey and Moore 1982; Lopez *et al.* 1989). However, this does not negate the importance of agrocin 84. Recent work has demonstrated that strain K84 produces at least two other antibiotics that exhibit inhibition specificities different from that of agrocin 84 (Donner *et al.* 1993; Maria Lopez, personal communication). These and other factors associated with strain K84 may well be responsible for the ability of this strain to control pathogens resistant to agrocin 84.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Strains of *A. tumefaciens, A. radiobacter,* and *E. coli* used in this study are listed in Table 3. Broad-host-range vector plasmids, recombinant clones, and the plasmids associated with the Tn*3*HoHo1 mutagenesis system (Stachel *et al.* 1985), along with their sources and characteristics, are listed

Table 3. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics[a]	Source or reference
Escherichia coli		
S17-1	*pro, recA, hsdR, thi,* Tp^r, Sm^r	Simon *et al.* 1983
HB101	F$^-$, *pro, leu, thi, recA, hsdR, hsdM*	Sambrook *et al.* 1989
DH5α	Φ80d*lacZ* Δ*M15,* Δ(*lacZYA-argF*), *U169, recA1, thi-1*	Sambrook *et al.* 1989
C2110	*polA*, Nal^r	Stachel *et al.* 1985
Agrobacterium radiobacter		
K84	pAgK84, pAtK84b, wild type	Kerr and Htay 1974
A. tumefaciens		
C58	pTiC58, wild type	Watson *et al.* 1975
K439	Tra-constitutive pTiC58, agrocin 84 supersensitive	Ellis *et al.* 1982
NT1	Ti plasmid-cured derivative of C58	Watson *et al.* 1975
A136	Rif^r, Nal^r mutant of NT1	Watson *et al.* 1975
Plasmids		
pAgK84	Production of and immunity to agrocin 84	Slota and Farrand 1982
pAgK84::Tn*5*-A1	Tn*5* insertion in pAgK84, Agn$^+$	Farrand *et al.* 1985
pAgK84::Tn*5*-A37	Tn*5* insertion in pAgK84, Agn$^+$	Farrand *et al.* 1985
pRK415	IncP1 broad-host-range cloning vector, Tc^r	Keen *et al.* 1988
pLAFR6	IncP1 broad-host-range cloning vector, Tc^r	Huynh *et al.* 1989
pHoHo1	Tn*3*HoHo1 donor plasmid, Cb^r	Stachel *et al.* 1985
pSShe	Tn*3*HoHo1 transposition helper plasmid	Stachel *et al.* 1985
pPH1JI	IncP1, Gm^r, Sp^r	Beringer *et al.* 1978
pBC4/5	pRK415::*Bam*HI C fragment from pAgK84, Tc^r	This study
pLA	pLAFR6::*Pst*I A fragment from pAgK84, Tc^r	This study
pLE13	pLAFR6::*Eco*RI GJK fragments from pAgK84, Tc^r	This study

[a] Agn$^+$, agrocin 84 production; Cb^r, carbenicillin resistance; Gm^r, gentamicin resistance; Nal^r, nalidixic acid resistance; Rif^r, rifampicin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Tc^r, tetracyline resistance; Tp^r, trimethoprim resistance.

in Table 3. Tn*5* insertion derivatives of pAgK84 used in the complementation analyses were described previously (Slota and Farrand 1982; Ryder *et al.* 1987). Insertion mutations in clones and marker exchange mutants in pAgK84 are described in the text and in Figures 1 and 2. An alternate Tn*3*HoHo1 delivery system was constructed by sequentially transforming pHoHo1 and pSShe into *E. coli* S17-1. This strain carries a copy of a modified RP1 integrated into its chromosome, and can directly mobilize many IncP1α and IncQ vectors (Simon *et al.* 1983).

Media and culture conditions.

Media used for the growth of *E. coli* and *Agrobacterium* strains were L broth, L agar, AB minimal medium, *vir* "induction medium" (Winans 1990), and Stonier's medium (Stonier 1960). Mannitol glutamate L broth (MG/L) (Cangelosi *et al.* 1992) was used for preparation of electrocompetent cells. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media at the following concentrations for *Agrobacterium:* carbenicillin (Cb), 100 μg/ml; gentamicin (Gm), 20 μg/ml; kanamycin, 50 μg/ml; rifampicin, 100 μg/ml; and tetracycline (Tc), 2 μg/ml. The following concentrations were used for *E. coli:* ampicillin (Ap), 100 μg/ml; kanamycin, 25 μg/ml; nalidixic acid, 20 μg/ml; and Tc, 10 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) was incorporated into indicator media at a final concentration of 40 μg/ml. Agrobacteria were grown at 28° C, and *E. coli* cultures were grown at either 28 or 37° C. All liquid cultures were incubated with vigorous agitation to ensure aerobic conditions. When required, growth was followed turbidimetrically with a Klett-Summerson colorimeter (red filter) or a Spectronic 20D spectrometer at 600 nm. Nopaline was purchased from Sigma, and a preparation of agrocinopines A and B was partially purified from *Euonymus* gall extracts as described previously (Ryder *et al.* 1987). To assess the influence of the carbon source on the induction of gene fusions, AB minimal medium was supplemented with the compound to be tested at a final concentration of 10 mM. Nopaline (1 mM) and agrocinopines A and B (25 μM) were added to AB mannitol minimal medium or to *vir* induction medium. Tobacco root exudate was prepared as described previously (Savka and Farrand 1992) and was added to AB mannitol minimal medium at a final concentration of 5% (v/v).

Gene transfer methods.

Bacterial matings were conducted on agar surfaces as described previously (Beck von Bodman *et al.* 1989). *Agrobacterium* transconjugants from matings with *E. coli* donors were selected on AB minimal medium plates containing sucrose as the sole source of carbon and the appropriate antibiotics. *E. coli* and *Agrobacterium* strains were transformed by the $CaCl_2$ technique (Sambrook *et al.* 1989) and the freeze-thaw technique (Holsters *et al.* 1978), respectively. Strains of *Agrobacterium* were rendered competent for electroporation as follows: 250-ml volumes of MG/L medium were inoculated with 1-ml volumes of an overnight culture of the bacterial strain. The cultures were incubated at 28° C with vigorous shaking until the OD_{600} reached a value of 0.3. The cultures then were transferred to cold centrifuge tubes and placed on ice for 15 min. Cells were collected by centrifugation at 7,000 rpm for 15 min at 4° C and resuspended in a volume of ice-cold buffer I (1% HEPES, pH 7.0) equal to half of the original culture volume. Cells were collected by centrifugation as above, and the pellet resuspended in a volume of ice-cold buffer II (1% HEPES and 10% glycerol, pH 7.0) equal to 1/50 of the original culture volume. Cells were collected again by centrifugation and resuspended in a volume of ice-cold buffer II equal to 1/250 of the original culture volume, and 0.1-ml aliquots were placed in sterile 1.5-ml microcentrifuge tubes and stored at –80° C until needed.

For electroporation, a tube of electrocompetent cells was thawed on ice, and the contents mixed gently with 1 μl of plasmid DNA. The cell suspension was transferred to a prechilled electroporation cuvette (0.2-cm gap) and pulsed in a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) at a voltage gradient of 12.5 kV/cm. After the pulse, 1 ml of MG/L broth was added immediately, and the cell suspension was transferred to a sterile culture tube. The cultures were incubated at 28° C with gentle shaking for 1 hr for recovery and expression prior to plating on selective media.

DNA manipulations.

Plasmid DNA was isolated and purified from *E. coli* and *Agrobacterium* cultures as described previously (Farrand *et al.* 1985). Restriction endonuclease digestions, agarose gel electrophoresis, DNA fragment purifications, and fragment religations all were performed by standard techniques (Sambrook *et al.* 1989). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (GIBCO BRL, Gaithersburg, MD); calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim (Indianapolis, IN).

Mutagenesis with Tn*3*HoHo1.

Fragments to be mutagenized were cloned into IncP1α vectors and transformed into *E. coli* S17-1(pHoHo1,pSShe). Transformants were selected on medium containing Tc and Ap, all of the colonies were pooled, and the mixed donor populations were mated *en masse* with *E. coli* C2110. Transconjugants were selected on L agar containing Ap and nalidixic acid. The plasmids in individual colonies were analyzed by restriction digestions, and the sites and polarities of the Tn*3*HoHo1 insertions were determined by comparing digests made with several different enzymes with those of the parent plasmid. Mutant plasmids of interest were transferred to *Agrobacterium* hosts by electroporation, selecting for resistance to Cb. In some cases, the C2110 transconjugants were pooled, and plasmid DNA was prepared and electroporated into *Agrobacterium* NT1. Electroporatants were selected on L agar containing Cb and X-Gal. Analysis of the individual plasmids, including mapping the sites of transposon insertions, was performed as described above.

Marker exchange mutagenesis.

Tn*3*HoHo1 insertions in cloned fragments were marker-exchanged into wild-type pAgK84 as follows. A recombinant plasmid with the insert of interest was mobilized from *E. coli* S17-1 to *A. tumefaciens* A136(pAgK84). *Agrobacterium* transconjugants were purified, and their plasmid content was verified by miniprep analysis. Marker-exchanged homogenotes were obtained by mating in the IncP1-incompatible

plasmid pPH1JI (Beringer *et al.* 1978). Transconjugants resistant to Cb and Gm but sensitive to Tc were identified on appropriate media and were screened by miniprep analysis for the absence of the recombinant clone bearing the transposon. The plasmid minipreps also were used to transform strain NT1. Selection for resistance to Cb yielded transformants harboring the marker-exchanged derivative of pAgK84 but lacking pPH1JI. All marker exchanges were verified by restriction endonuclease digestions of purified plasmid DNA isolated from these final transformants.

Agrocin 84 assays.

Production of agrocin 84 was determined on Stonier's agar plates by the soft agar overlay method essentially as described by Hayman and Farrand (1988), with *A. tumefaciens* C58 as the susceptible indicator. In some cases, strain K439, which is supersensitive to agrocin 84 (Ellis *et al.* 1982), also was used. In some experiments the assay was modified as follows: 1 ml of an overnight culture of the indicator strain was mixed with 13 ml of Stonier's medium containing 0.7% agar and poured into a petri dish. After the agar had gelled, strains to be tested for production were spotted directly onto the surface of the medium. Plates were examined for zones of growth inhibition after 24–48 hr of incubation at 28° C. When strain K84 was used as the producer, the medium was supplemented with biotin (0.2 μg/ml).

β-Galactosidase assays.

While biovar 2 agrobacteria, strain K84 included, exhibit a strong β-galactosidase activity (Williams *et al.* 1992), biovar 1 strains of *Agrobacterium* catabolize lactose via the 3-ketolactose pathway (Bernaerts and De Ley 1963) and do not normally express any endogenous X-Gal$^+$ activities. For this reason, all experiments involving analysis of fusions with *lacZ* were performed with NT1 or its derivatives. Production of β-galactosidase was qualitatively assessed on solid media supplemented with X-Gal. β-Galactosidase levels in cells were quantitated by the method described by Stachel *et al.* (1985) except that activity was expressed per 10^9 cfu rather than as a function of OD_{600}. For the time-course assay, 0.2 ml of cell culture was removed at the indicated time intervals. Otherwise, 0.2-ml portions of cell suspension were removed when cells reached the late exponential growth stage. All samples were frozen at −80° C, and when sampling was completed, all were thawed, and β-galactosidase activities were assayed.

***In planta* assays.**

Tomato seeds (cultivar Rutgers 39 VF) were germinated aseptically on water agar plates. The seedlings were inoculated by dipping the root systems into a suspension of an overnight culture of the bacterial strain to be tested (approximately 10^9 cfu/ml). The inoculated seedlings were planted in separate pots containing a sterile mixture of perlite, vermiculite, and sand (2:1:1) and grown under greenhouse conditions. After 2 wk, the plants were removed from the pots, adherent soil was removed by gentle shaking, and the root systems were dissected from the stems. Each root system was dipped briefly in 1.0 ml of 0.85% NaCl to remove remaining soil particles and then was transferred to a tube containing 1.0 ml of 0.85% NaCl. The root systems were washed by vortexing three times over 5-min intervals. The roots were removed, and suspended soil particles, intact plant cells, and plant cell debris were removed by centrifugation for 5 min at 175 × *g* in an HR4 rotor of a Sorvall GLC-2B centrifuge at room temperature. A small volume of the resulting supernatant was removed for enumeration of the bacteria by dilution plate counting. The remainder was used to assay for β-galactosidase levels, and activity was expressed per 10^9 cfu. Plant roots inoculated with a strain harboring pAgK84::Tn*5*-A1, which does not contain a *lacZ* fusion, were processed in an identical manner. β-Galactosidase activity levels determined from bacterial suspensions obtained from these roots served as a control for enzyme activity contributed by the tomato plant. These values were always less than 10% of those from strains containing *lacZ* fusions.

ACKNOWLEDGMENTS

This work was supported by grant 89-37263-4754 from the U.S. Department of Agriculture to S.K.F.

LITERATURE CITED

Alconero, R. 1980. Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. Plant Dis. 64:835-838.

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

Beringer, J. E., Beynon, J. L., Buchanan-Wollaston, A. V., and Johnston, A. W. B. 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature 276:633-634.

Bernaerts, M. J., and De Ley, J. D. 1963. A biochemical test for crown gall bacteria. Nature (London) 197:406-407.

Cangelosi, G. A., Best, E. A., Martinetti, G., and Nester, E. W. 1992. Methods for genetic analysis of *Agrobacterium*. Methods Enzymol. 204:384-397.

Cooksey, D. A., and Moore, L. W. 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. Phytopathology 72:919-921.

Dessaux, Y., Petit, A., and Tempé, J. 1992. Opines in *Agrobacterium* biology. Pages 109-136 in: Molecular Signals in Plant-Microbe Communications. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.

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

Donner, S. C., Jones, D. A., McClure, N. C., Rosewarne, G. M., Tate, M. E., Kerr, A., Fajardo, N. N., and Clare, B. G. 1993. Agrocin 434, a new plasmid encoded agrocin from *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria. Physiol. Mol. Plant Pathol. 42:185-194.

Ellis, J. G., Kerr, A., van Montagu, M., and Schell, J. 1979. *Agrobacterium*: Genetic studies on agrocin 84 production and the biological control of crown gall. Physiol. Plant Pathol. 15:311-319.

Ellis, J. G., Murphy, P. J., and Kerr, A. 1982. Isolation and properties of transfer regulatory mutants of the nopaline Ti plasmid of pTiC58. Mol. Gen. Genet. 186:275-281.

Farrand, S. K. 1991. Biological control of microbial plant pathogens through antibiosis. Pages 311-329 in: CRC Pest Management Handbook. Vol. 2. 2nd ed. D. Pimentel, ed. CRC Press, Boca Raton, FL.

Farrand, S. K., O'Morchoe, S. P., and McCutchan, J. 1989. Construction of an *Agrobacterium tumefaciens* C58 *recA* mutant. J. Bacteriol. 171: 5314-5321.

Farrand, S. K., Slota, J. E., Shim, J.-S., and Kerr, A. 1985. Tn*5* insertions in the agrocin 84 plasmid: The conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. Plasmid 13:106-117.

Farrand, S. K., Wang, C.-L., Hong, S.-B., O'Morchoe, S. B., and Slota, J. E. 1992. Deletion derivatives of pAgK84 and their use in the analy-

sis of *Agrobacterium* plasmid function. Plasmid 28:201-212.
Formica, J. V. 1990. Conditions for the production of agrocin 84 by *Agrobacterium radiobacter* K84. J. Ind. Microbiol. 5:33-44.
Gutterson, N., Ziegle, J. S., Warren, G. J., and Layton, T. J. 1988. Genetic determinants for catabolite induction of antibiotic biosynthesis in *Pseudomonas fluorescens* Hv37. J. Bacteriol. 170:380-385.
Hayman, G. T., and Farrand, S. K. 1988. Characterization and mapping of the agrocinopine–agrocin 84 locus on the nopaline Ti plasmid pTiC58. J. Bacteriol. 170:1759-1767.
Holsters, M., DeWaele, D., Depicker, A., Messens, E., van Montagu, M., and Schell, J. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol. Gen. Genet. 163:181-187.
Hong, S.-B., Dessaux, Y., Chilton, W. S., and Farrand, S. K. 1993. Organization and regulation of the mannopine cyclase-associated opine catabolism genes in *Agrobacterium tumefaciens* 15955. J. Bacteriol. 175:401-410.
Howie, W. L., and Suslow, T. V. 1991. Role of antibiotic biosynthesis in the inhibition of *Pythium ultimum* in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescens*. Mol. Plant-Microbe Interact. 4:393-399.
Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.
Keen, N. T., Tamaki, S., Kobayashi, D., and Trolliger, D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70:191-197.
Kennedy, B. W., and Alcorn, S. M. 1980. Estimates of U.S. crop losses to procaryote plant pathogens. Plant Dis. 64:674-676.
Kerr, A., and Htay, K. 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. 4:37-44.
Lopez, M. M., Gorris, M. T., Salcedo, C. I., Montojo, A. M., and Miro, M. 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. Appl. Environ. Microbiol. 55: 741-746.
Moore, L. W., and Warren, G. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Annu. Rev. Phytopathol. 17: 163-179.
Murphy, P. J. 1982. Agrocin 84 sensitivity and toxicity in *Agrobacterium*. Ph.D. thesis, University of Adelaide, Adelaide, South Australia.
Murphy, P. J., Tate, M. E., and Kerr, A. 1981. Substituents at N^6 and C-5′ control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in agrobacteria. Eur. J. Biochem. 115:539-543.
Nautiyal, C. S., Dion, P., and Chilton, W. S. 1992. Diauxic growth of *Agrobacterium tumefaciens* 15955 on succinate and mannopine. J. Bacteriol. 174:2215-2221.
Piper, K. P., Beck von Bodman, S., and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature 362:448-450.
Ryder, M. H., Slota, J. E., Scarim, A., and Farrand, S. K. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. J. Bacteriol. 169:4184-4189.
Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Savka, M. A., and Farrand, S. K. 1992. Mannityl opine accumulation and exudation by transgenic tobacco. Plant Physiol. 98:784-789.
Schroth, M. N., and Hancock, J. G. 1981. Selected topics in biological control. Annu. Rev. Microbiol. 35:453-465.
Simon, R. U., Prifer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in gram-negative bacteria. Biotechnology 1:784-791.
Slota, J. E., and Farrand, S. K. 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin production. Plasmid 8:175-186.
Stachel, S. E., An, G., Flores, C., and Nester, E. W. 1985. A Tn*3lacZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium*. EMBO J. 4:891-898.
Stonier, T. 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. J. Bacteriol. 79:889-898.
Tate, M. E., Murphy, P. J., Roberts, W. P., and Kerr, A. 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature 280:697-699.
Thomashow, L. S., Weller, D. M., Bonsall, R. F., and Pierson, L. S., III. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56:908-912.
Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D., and Nester, E. W. 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. 123:255-264.
Williams, S. G., Greenwood, J. A., and Jones, C. W. 1992. Molecular analysis of the *lac* operon encoding the binding-protein-dependent lactose transport system and β-galactosidase in *Agrobacterium radiobacter*. Mol. Microbiol. 6:1755-1768.
Winans, S. C. 1990. Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation, and acidic growth media. J. Bacteriol. 172:2433-2438.
Winans, S. C. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. 56:12-31.
Zambryski, P. C. 1992. Chronicles from the *Agrobacterium*–plant cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 465-490.