Opine Catabolic Loci from *Agrobacterium* Plasmids Confer Chemotaxis to Their Cognate Substrates

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Opines are carbon compounds produced by crown galls and hairy roots induced by *Agrobacterium tumefaciens* and *A. rhizogenes*, respectively. These novel condensation products of plant metabolic intermediates are utilized as nutritional sources by the *Agrobacterium* strains that induced the growths. Thus, opines are thought to favor the propagation of agrobacteria in the tumorsphere. Certain *Agrobacterium* strains were chemoattracted to opines. The chemotactic activities to octopine, to nopaline, to mannopine, and to agrocinopines A+B were dependent on the type of the Ti plasmid present in the bacterium. The determinants for chemotaxis to these opines were localized to the regions of the octopine- and nopaline-type Ti plasmids coding for transport and catabolism of that opine. An insertion in *accA*, which encodes the periplasmic binding protein for agrocinopines A+B, abolished chemotaxis while an insertion in *accC*, which encodes a component of the transport system, and an insertion in *accF*, which encodes a function required for agrocinopine catabolism, did not affect chemotaxis to this opine. Thus, transport and catabolism of these opines are not required for the chemotactic activity. Analyses of subclones of the *acc* region confirmed that *accA* is the only gene required from the Ti plasmid for chemotaxis to agrocinopines A+B.

Additional keywords: ABC-type transport system, *moc*, *noc*, *occ*.

Agrobacterium tumefaciens causes crown gall disease on many dicotyledonous plants by transferring a small piece of DNA from the bacterium to the plant. The transferred DNA or T-DNA is a part of a large extra chromosomal element called the Ti plasmid (Chilton et al. 1980, 1982). Once integrated into the genome of the susceptible plant, a number of genes encoded by the T-DNA are expressed (Van Onckelen et al. 1986; Watson et al. 1975). One set of genes codes for the biosynthesis by the tumors of novel metabolites called opines (Dessaux et al. 1992; Ellis and Murphy 1981). Most opines are imine conjugates of ketoacids and sugars or amino acids, although two families are composed of sugars linked by a

phosphodiester bond (Dessaux et al. 1992). The opines are specific nutritional resources for agrobacteria. Some also act as signals that are required for induction of conjugal transfer of Ti plasmids from a donor bacterium to a recipient (Beck von Bodmann et al. 1989; Ellis et al. 1982).

Most known Ti plasmids that contain opine synthesis genes on their T-DNAs also encode a set of corresponding non-transferred genes that confer catabolism of that same opine on the bacterium (Bomhoff et al. 1976; Hayman et al. 1993; Kim and Farrand 1997; Zanker et al. 1992). This correlation between opine synthesis in tumors and opine degradation by the bacteria suggests that opine production by crown galls may provide the pathogen with selective growth substrates favoring its propagation (Guyon et al. 1993; Schell et al. 1979; Tempé et al. 1979). Thus, Tempé et al. (1979) proposed that opines are the chemical mediators of the interaction between *Agrobacterium* spp. and their plant host, a concept supported by recent experimental evidence (Guyon et al. 1993; Wilson et al. 1995; Oger et al. 1997; Savka and Farrand 1997).

Agrobacteria are chemoattracted to a wide range of amino acids, phenolic compounds, and sugars, some of which are known *vir* gene inducers (Cangelosi et al. 1990; Loake et al. 1988; Parke et al. 1987). Thus, it is plausible that chemotaxis of the bacteria to phenolic compounds and sugars released by plants may be one mechanism by which the bacteria recognize wound sites during the initial infection process. The chemotactic activity of these compounds apparently is encoded by the chromosome (Cangelosi et al. 1990; Hawes et al. 1988; Loake et al. 1988; Parke et al. 1987). However, there also are reports that *A. tumefaciens* strain C58 requires the Ti plasmid-encoded *virA* and *virG* genes for chemotaxis to phenolic compounds with *vir* gene inducer activity (Ashby et al. 1988; Shaw et al. 1988; Shaw 1991).

To our knowledge, the chemotactic properties of opines for agrobacteria have not been investigated. Here, we show that some opines are chemoattractants, and that this chemotaxis is specific to the type of Ti or opine catabolic plasmid present in the cell. In addition, we show that chemotaxis to opines requires functions encoded by the opine catabolism operons of the *Agrobacterium* plasmids.

RESULTS

Agrobacteria are attracted to opines.

We tested four opines that are found in galls induced by nopaline- or by octopine-type *A. tumefaciens* strains for their

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ability to chemoattract agrobacteria. We used two assays based on motility in soft agar. In the directional swarm plate assay, chemotaxis is indicated by an arc of bacterial growth, facing toward the spot at which the test substrate was applied at the edge of the plate. In the classic swarm plate assay developed by Adler (Adler 1966; Armstrong et al. 1967), chemotaxis is indicated by expanding rings of bacterial growth on the plates that contain an appropriate substrate as sole carbon source. We used this assay to confirm some of the results from the directional swarm plate assay. Strains NT1 and SA122, Ti plasmidless derivatives of the nopaline-type strain C58 and the octopine-type strain 15955, respectively, were not attracted to any of four opines tested, but were strongly attracted to sucrose (Figs. 1 and 2; Table 1). Nopaline, agrocinopines A+B, mannopine, and octopine chemoattracted motile *A. tu-*

meffaciens strains. Chemotaxis to mannopine and to octopine was generally more obvious when tested by the classic swarm plate assay than by the directional swarm plate assay (data not shown). Chemotactic responses toward nopaline and agrocinopines A+B were stronger than that toward mannopine or to octopine. Attraction was associated with the presence of the cognate Ti plasmid for that particular opine. Strain C58 was attracted to agrocinopines A+B and to nopaline but not to octopine or to mannopine, and strain 15955 was attracted to octopine and to mannopine but not to agrocinopines A+B or to nopaline (Table 1). Another octopine-type strain, R10, was not motile under any conditions tested. Interestingly, the non-tumorigenic biocontrol strain, *Agrobacterium radiobacter* K84, also was attracted to agrocinopines A+B and to nopaline (Table 1).

Fig. 1. Directional swarm plate assays, conducted as described in Materials and Methods. Arrowheads denote sites at which opines were spotted onto the agar at the edges of the plates. **A**, Strain C58 shows an arc of bacterial growth facing toward the site at which agrocinopines were spotted, while strain NT1, a Ti plasmidless derivative of strain C58, does not produce such an arc. **B**, Assays with the same set of strains but with nopaline. Strain C58 shows an arc of bacterial growth, while strain NT1 does not. Both strains show the classic swarming motility with concentric rings associated with chemotaxis to mannitol, the carbon source included in the medium.

Fig. 2. Classic swarm plate assays demonstrating chemotaxis to octopine, to mannopine, and to nopaline. Assays were conducted as described in Materials and Methods. **A**, Strain 15955 shows concentric rings of swarming growth from the point of inoculation in the medium containing octopine, while strain SA122, a Ti plasmidless derivative of strain of 15955, does not produce such a ring. **B**, Assays with the same set of strains but with mannopine. Strain 15955 shows rings of swarming bacterial growth, while strain SA122 does not. **C**, assays of strains C58 and SA122(pTiC58) with nopaline. C58 shows the concentric rings of bacterial growth, while strain SA122(pTiC58) does not.

Ti plasmids code for determinants for chemotaxis to opines.

Strains NT1 or SA122 harboring octopine-type Ti plasmids pTiR10 or pTi15955 were attracted to octopine and to mannopine, but not to agrocinopines A+B or to nopaline (Table 1). Similarly, NT1 harboring the nopaline-type Ti plasmid pTiC58 was attracted to agrocinopines A+B and to nopaline, but not to octopine or to mannopine (Table 1). However, SA122 harboring pTiC58 was not attracted to any of these opines (Table 1; Fig. 2C). These results indicate that Ti plasmids are required for attraction to opines and that each Ti plasmid codes for chemotaxis determinants that are specific for the opines to which the plasmid confers utilization. However, our results also indicate that, at least for pTiC58, a different chromosomal background can affect chemotaxis to nopaline (Table 1; Fig. 2C).

Chemotaxis to opines also is associated with pAtK84b, the large plasmid from strain K84 that codes for catabolism of nopaline and agrocinopines A+B (Hayman and Farrand 1990; Tate et al. 1979). This plasmid is very closely related to pTiC58, but lacks a T region and the entire *vir* regulon (Clare et al. 1990). Like strain K84, strain NT1(pAtK84b) was strongly attracted by both of these opines but not by octopine or mannopine (Table 1). This also indicates that pAtK84b confers chemotaxis to opines equally well in both the biovar 2 K84 and the biovar 1 C58 chromosomal backgrounds.

Chemotaxis requires functions encoded by the opine catabolism operons.

Various cosmid clones containing inserts of different regions of octopine- and nopaline-type Ti plasmids and of pAtK84b were tested in NT1 or in SA122 to localize the determinants for chemotaxis to opines. pTHH6, pTHB112, pYDH208, and pYDH501 are Ti plasmid clones that code for the opine catabolic operons *noc*, *acc*, *moc*, and *occ*, respectively (Fig. 3; Table 2; Hong and Farrand 1993; Kim and Farrand 1997; Zanker et al. 1992). Derivatives of strain NT1 harboring such cosmid clones were chemoattracted to the cognate opine but not to opines of which the clone does not confer catabolism, with one exception: NT1(pYDH501), which codes

for a mannopine transport system but lacks genes for catabolism of this opine (Farrand et al. 1996b; Oger et al., *in press*), also conferred weak attraction to mannopine (Table 2). While the directional swarm plate assay showed chemotactic behavior of NT1(pYDH501) to mannopine, the classic swarm plate assay, because it requires utilization of the test compound, failed to show this attraction (data not shown). A derivative of strain SA122 harboring pYDH501 showed much stronger chemotaxis toward octopine than did NT1 harboring the same plasmid (data not shown). Randomly chosen clones not known to encode catabolism or transport functions for opines did not confer chemotaxis to octopine, nopaline, mannopine, or agrocinopines A+B in strain NT1 (Fig. 3 and data not shown).

Cosmid clones pBC201 and pBC382 containing inserts from pAtK84b conferred chemotaxis to nopaline and to agrocinopine opines, respectively (Table 2). pBC201 contains an insert that shows strong homology to *noc* of pTiC58 (Clare et al. 1990). However, while NT1(pBC201) grew on medium containing nopaline as sole carbon source, it did so at a rate much slower than that of NT1(pAtK84b), NT1(pTiC58), or NT1(pTHH6) (data not shown). By hybridization analysis, the insert in pBC382 shows only very weak relatedness with *acc* of pTiC58 (Clare et al. 1990; Hayman and Farrand 1990) but maps to a position on pAtK84b similar to that at which *acc* is located on pTiC58. This clone allowed NT1 to take up agrocinopines A+B from the culture medium (Fig. 4).

A clone conferring uptake of mannopine elicits an unusual response in the chemotaxis assay.

We tested various subclones of the *moc* region of the octopine-type Ti plasmid pTi15955 for their ability to confer chemotaxis to mannopine. NT1 harboring pRE10, which constitutes a part of pYDH208, showed the arc of bacterial growth toward mannopine, as does NT1 harboring pYDH208 (Table 2). On the other hand, we observed that pDSRE10a, which contains the whole *Eco*RI fragment 10 cloned in the IncQ vector pDSK519, induced an unusual arcing of cells within the growth zone on the directional swarm assay plates (Fig. 5). This phenomenon was specifically associated with

Table 1. Chemotaxis to opines by *Agrobacterium* strains containing different plasmids

Strain	Type of plasmid	Opine ^a				
		Agrocinopines ^b	Nopaline ^c	Octopine ^c	Mannopine ^c	Sucrose ^c
15955	Ti, <i>occ/moc</i>	—	—	+	+	+
R10 ^d	Ti, <i>occ/moc</i>	—	—	—	—	—
C58	Ti, <i>noc/acc</i>	+	+	—	—	+
K84	At, <i>noc/acc</i>	+	+	—	—	+
NT1	None	—	—	—	—	+
SA122	None	—	—	—	—	+
NT1(pTiC58)	Ti, <i>noc/acc</i>	+	+	—	—	+
SA122(pTiC58)	Ti, <i>noc/acc</i>	—	—	—	—	+
NT1(pTiR10)	Ti, <i>occ/moc</i>	—	—	+	+	+
NT1(pTi15955)	Ti, <i>occ/moc</i>	—	—	+	+	+
SA122(pTi15955)	Ti, <i>occ/moc</i>	—	—	+	+	+
NT1(pAtK84b)	At, <i>noc/occ</i>	+	+	—	—	+
NT1(pAgK84b)	Agrocin	—	—	—	—	+

^a Chemotaxis assays were done as described in Materials and Methods. +, positive chemotaxis toward opine; —, no chemotaxis toward opine observed.

^b Chemotaxis to agrocinopines was assessed by the directional swarm plate assay only.

^c Chemotaxis to nopaline, to octopine, to mannopine, and to sucrose was tested by the classic swarm plate assay and the directional swarm plate assay as described in Materials and Methods.

^d Strain R10 is not motile.

mannopine; sugars or other opines did not induce this effect (data not shown). Clones pRE10 and pYDH208, which contain all but the most distal part of *Eco*RI fragment 10, did not elicit this unusual growth effect (Fig. 5). NT1 harboring pDSRE10b, in which *Eco*RI fragment 10 is cloned in the opposite orientation with respect to the *lac* promoter of the vector, also did not produce this arc (data not shown).

Chemotaxis to octopine by mutants that contain insertions in *occ*.

Mutations in the *occ* region of an octopine-type Ti plasmid, pTiR10, were tested in strain SA122 for their effect on chemotaxis to octopine. Insertions in *occM* and *occQ*, which code

for the integral membrane components of the octopine transport system, abolished chemotaxis to octopine. However, insertions in *ooxA* and *ooxB*, which encode the two subunits of octopine oxidoreductase (Cho et al. 1996; Zanker et al. 1992), did not affect chemotaxis to octopine (Table 3) in the directional swarm plate assay.

accA from pTiC58 is required for chemotaxis to agrocinopines A+B.

The *occ*, *noc*, *moc*, and *acc* operons all encode ABC-type transporters (Valdivia et al. 1991; Zanker et al. 1992; Farrand et al. 1996b; Kim and Farrand 1997; Oger et al., *in press*). The periplasmic binding proteins associated with these types of

Fig. 3. Genetic and physical organization of nopaline- and octopine-type Ti plasmids. **A**, Map of pTiC58. **B**, Map of pTi15955. Ti plasmid regions contained in the cosmid clones used for the assays are shown as arcs outside of the circular maps. Filled arcs denote clones that encode opine catabolism that confer chemotaxis. Ti plasmid regions of known functions are shown: *acc*, agrocinopine catabolism; *noc*, nopaline catabolism; *occ*, octopine catabolism; *moc*, mannitol opine catabolism; *vir*, T-DNA transfer to plants; *tra* and *trb*, conjugal transfer of the Ti plasmid; *ape*, bacteriophage API exclusion; *rep/inc*, vegetative replication of the Ti plasmid and plasmid incompatibility.

Table 2. Chemotaxis to opines by *Agrobacterium* strains harboring different cosmid clones

Strain	Source of clone	Opine utilized	Opine ^a			
			Agrocinopines	Nopaline	Octopine ^b	Mannopine ^b
NT1	—	None	—	—	—	—
SA122	—	None	—	—	—	—
NT1(pTHB112)	pTiC58	Agrocinopine	+	—	—	—
NT1(pTHH6)	pTiC58	Nopaline	—	+	—	—
NT1(pTHH17)	pTiC58	None	—	—	—	—
NT1(pTHH23)	pTiC58	None	—	—	—	—
NT1(pYDH501)	pTi15955	Octopine	—	—	+	—
NT1(pYDH304)	pTi15955	MOA, AGA	—	—	—	—
NT1(pYDH208)	pTi15955	mannopine	—	—	—	+
SA122(pYDH501)	pTi15955	Octopine	—	—	+	—
SA122(pTHH23)	pTi15955	None	—	—	—	—
SA122(pYDH304)	pTi15955	MOA, AGA	—	—	—	—
SA122(pYDH208)	pTi15955	mannopine	—	—	—	+
NT1(pRE10)	pTi15955	None	—	—	—	+
NT1 (pBC201)	pAtK84b	Nopaline ^c	—	+	—	—
NT1 (pBC382)	pAtK84b	ND ^d	+	—	—	—

^a Chemotaxis assays were done as described in Materials and Methods. +, positive chemotaxis toward opine; —, no chemotaxis toward opine observed.

^b Chemotaxis to octopine and to mannopine were tested by both the classic swarm plate assay and the directional swarm plate assay as described in Materials and Methods.

^c NT1(pBC201) grew on medium containing nopaline as sole carbon source but at a rate slower than that of NT1(pAtK84b), or NT1(pTiC58).

^d Not determined.

transporters often serve as primary receptors for chemotaxis to the substrate. For recent reviews, see Higgins (1992) and Stock and Surette (1996). Cosmid clone pTHB112, derived from pTiC58, contains the entire *acc* operon, and codes for all of the determinants required for transport and catabolism of agrocinopines A+B, as well as for growth with these opines as sole carbon source (Hayman et al. 1993; Kim and Farrand 1997). Different subclones and Tn3HoHo1 insertion mutants of this clone were tested for their ability to confer chemotaxis to agrocinopines A+B (Fig. 6). Among the Tn3HoHo1 insertions spanning pTHB112, only insertion 476, located in *accA*, totally abolished chemotaxis to agrocinopines A+B. A Tn3HoHo1 insertion in *accC*, which codes for a component of the transport system, and an insertion mutation in *accF*, which encodes a function required for catabolism of agrocinopines A+B (Kim and Farrand 1997), did not affect chemotaxis to these opines (Fig. 6). Derivatives of pTiC58 in which each of the mutations in *acc* were marker-exchanged into the Ti plasmid gave results indistinguishable from those observed with the clones (data not shown). Insertion 153, located in *accR*, which encodes the repressor involved in regulating expression of *acc* (Beck von Bodmann et al. 1992; Kim and Farrand 1997), weakened but did not completely abolish chemotaxis to agrocinopines A+B (data not shown). Similarly, pTiC58 Δ *accR*, which contains a 5-bp deletion in *accR* and constitutively expresses *acc* (Beck von Bodmann et al. 1992), conferred weakened chemotaxis to agrocinopines A+B (data not shown). Neither mutation in *accR* disrupted motility or chemotaxis to mannitol or sucrose (data not shown). Nor did the Δ *accR* mutation in pTiC58 have any effect on chemotaxis to nopaline. We examined the possibility that *accR* might play a direct role in chemotaxis to agrocinopines by assessing chemotactic responses of an *accC* mutant with and without a mutation in *accR*. Strain NT1K438, which harbors pTiC58 Δ *accR* with a mutation in *accC*, showed about the

same strength of chemotaxis to agrocinopines A+B as did strain C58K438, which harbors pTiC58 with the same *accC* mutation (data not shown).

Subclones of pTHB112 that contain *accA* in the insert, including pTHSB5, pTHSS9, pDS3.1, pTHH2, and pTH32, conferred chemotaxis to agrocinopines A+B (Fig. 6). On the other hand, clones that do not contain an intact *accA*, such as pTHH7 and pTHE16, failed to confer chemotaxis to these opines. pDS3.1, the smallest of the clones that confer chemotaxis to the agrocinopines, contains only two genes of the *acc* operon, *accA* and *accB*. pTHSS9, which codes for all of *accA* but a truncated *accB*, also confers chemotaxis to agrocinopines, indicating that an intact *accB* is not required for attraction.

DISCUSSION

Using two swarm plate assays, one as described by Adler (1966), the other modified from the work of Hawes et al. (1988), we show that certain opines are chemoattractants to their cognate *Agrobacterium* strains. The determinants conferring specificity for chemotaxis to these substrates are encoded by the large plasmids present in these strains, and not by the chromosome. Our genetic analysis indicates that the Ti plasmid functions required for chemotaxis are associated with the regions encoding catabolism of that opine. However, at least for the agrocinopines, opine transport and catabolism are not required for chemotaxis (Fig. 5). This is consistent with other substrate-associated chemotaxis systems (Abouhamad et al. 1991). In addition, this confirms the validity of the directional plate assay; the arc-shaped swarm of bacteria did not form due to promoted growth of the bacteria by the opine spotted at the edge of the plates. The classic swarm plate assay, on the other hand, requires utilization of the substrate by the tested bacteria to demonstrate the chemotactic behavior. Thus, this assay cannot be used to test chemotaxis toward nonutilizable sub-

Fig. 4. Uptake of agrocinopines A+B by NT1 harboring clones of pAtK84b. Samples from the cell suspensions were taken at 0 and at 48 h after addition of the opines, the cells were removed by centrifugation, and the supernatants were subjected to high voltage paper electrophoresis (HVPE) in formic acid-acetic acid buffer, as described in Materials and Methods. Ori, origin of electrophoresis. Orange G and xylene cyanol are used as electrophoretic mobility markers.

strates. In this classic swarm plate assay, utilizable but non-chemoattracting substrates result in a slowly forming diffuse radiation of bacterial growth without obvious concentric rings. This was the type of reaction we observed for strain SA122 harboring pTiC58 against nopaline (Fig. 2C). This strain also failed to give an arc toward nopaline in the directional swarm plate assay (Table 1).

Octopine, nopaline, mannopine, and the agrocinopines are taken up by ABC-type transporters (Valdivia et al. 1991; Zanker et al. 1992; Kim and Farrand 1997; Farrand et al. 1996b; Oger et al., *in press*). These systems from gram-negative bacteria all contain, in addition to the membrane-associated permeases, a substrate-binding protein located in the periplasm. AccA (Hayman et al. 1993) is located in the

periplasm of bacteria expressing the appropriate genes. Our analyses indicate that *accA*, the gene encoding AccA, is sufficient to confer chemotaxis to the agrocinopines. On the other hand, insertion mutations in genes coding for the trans-membrane and energizer components of the agrocinopine transporter have no effect on chemotaxis (Fig. 6). In contrast, insertions in *occM* and *occQ*, coding for the trans-membrane components of the octopine transporter, abolished chemotaxis to octopine (Table 3). This probably is due to polar effects of these insertions on *occJ*, which is the distal gene of the transport cluster and codes for the periplasmic octopine-binding protein OccJ (Valdivia et al. 1991). Insertions in *ooxA* and *ooxB*, encoding octopine oxidoreductase, did not affect chemotaxis to octopine, as determined by the directional swarm

Fig. 5. Directional swarm plate assays with mannopine of NT1 harboring pDSRE10a or pRE10. **A**, Restriction map of the pTi15955 region that encodes the mannopine transport system, *trlR*, and *mcl* (Oger et al., *in press*). Inserts present in the plasmids pDSRE10a(b), pYDH208, and pRE10 are shown above corresponding regions of the map. **B**, Strain NT1(pDSRE10a), which has the entire *EcoRI* fragment 10, shows an unusual pattern of arcing. Strain NT1(pRE10), which does not have the distal portion of the *EcoRI* fragment 10, does not produce such growth. A similar lack of response was observed for NT1(pDSRE10b) in which the insert in the clone is oriented opposite to the direction of the *lac* promoter. Assays were conducted as described in Materials and Methods and in Figure 1. Arrowheads denote sites at which mannopine was spotted.

plate assay (Table 3), ruling out the need for catabolic functions and supporting the hypothesis that *occJ* is required for chemotaxis to octopine.

These results with mutants of agrocinopine and octopine utilizing systems are consistent with observations in other

systems. Periplasmic binding proteins associated with ABC-type transporters often function as chemotaxis receptors independent of their transporter activities. Moreover, in *A. tumefaciens*, ChvE, a chromosomally-encoded periplasmic sugar-binding protein, mediates chemotaxis to several sugars, as well as their uptake via an ABC-type transporter (Cangelosi et al. 1990; Huang et al. 1990; Kemner et al. 1997). Interestingly, ChvE also is required for maximum induction of the Ti plasmid *vir* regulon in a synergistic signaling interaction that requires phenolic inducers and any one of several sugar ligands that can bind to the periplasmic protein (Cangelosi et al. 1990; Huang et al. 1990). This protein when bound with its ligand evidently can interact with the periplasmic domain of VirA in a manner analogous to that by which these periplasmic binding proteins interact with chemotaxis sensor proteins.

We propose that AccA, and probably OccJ, NocT, the putative periplasmic binding protein of *noc* (Zanker et al. 1992), and MotA, the putative periplasmic mannopine-

Table 3. Chemotaxis to octopine by strain SA122 harboring pTiR10 with mutations in the *occ* operon

Strain ^a	Affected gene	Chemotaxis to octopine ^b
SA122	None	-
SA122(pTiR10-11)	<i>occQ</i>	-
SA122 (pTiR10-17)	<i>occM</i>	-
SA122 (pTiR10-8)	<i>ooxB</i>	+
SA122 (pTiR10-16)	<i>ooxA</i>	+

^a pTiR10 containing insertion mutations in *occ* was electroporated into strain SA122 as described in Materials and Methods.

^b Chemotaxis assays were done by directional swarm plate assay as described in Materials and Methods. +, positive chemotaxis toward opine; -, no chemotaxis toward opine observed.

Fig. 6. Chemotaxis to agrocinopines A+B by strain NT1 harboring pTHB112, and various Tn3HoHo1 insertion mutants and subclones of this cosmid clone. Tn3HoHo1 insertions are shown as vertical lines (Hayman et al. 1993; Kim and Farrand 1997). Crossbars on each insertion indicate the transcriptional orientation of the Tn3HoHo1 *lacZ* gene. Genes in the *acc* operon are abbreviated as follows: R, *accR*; A, *accA*; B, *accB*; C, *accC*; D, *accD*; E, *accE*; F, *accF*; G, *accG*. Chemotaxis to agrocinopines A+B, determined by the directional swarm plate assays as described in Materials and Methods, is indicated as (+) or (-) above each Tn3HoHo1 insertion or each restriction fragment depicted. Each Tn3HoHo1 insertion has the prefix pTHB- (Hayman et al. 1993), or pHK- (Kim and Farrand 1997).

binding protein (Farrand et al. 1996b; Oger et al., *in press*), function as primary receptors for chemotaxis to their cognate opines. In *Escherichia coli* and *Salmonella typhimurium* the periplasmic binding protein-ligand complexes interact with the periplasmic domain of integral membrane chemotaxis receptors called methyl-accepting chemotaxis proteins (Mcps; Stock and Surette 1996). These receptors are coded for by genes located on the bacterial chromosome. Since AccA, and probably the other periplasmic opine-binding proteins as well, are the only Ti plasmid-encoded proteins required for chemotaxis to their cognate opines, it seems likely that they can interact with Mcps expressed from genes located on the *Agrobacterium* chromosome. Interestingly, SA122 harboring pYDH501, a cosmid clone of the octopine-type Ti plasmid pTi15955, exhibits much stronger chemotaxis to octopine than does NT1 harboring the same plasmid (data not shown). Similarly, NT1 harboring pTiC58 exhibits chemotaxis to agrocinopines and to nopaline, while SA122 containing the same plasmid failed to show chemotaxis to these opines (Fig. 2C and data not shown). These data indicate that OccJ and AccA most likely interact with the Mcps expressed from genes located on the *Agrobacterium* chromosome, and that these periplasmic opine-binding proteins may have higher affinity to the Mcps from their natural hosts than to those from the other opine-type *Agrobacterium* strains tested. Intriguingly, our recent sequence analysis of a region of pTiC58 adjacent to *acc* indicates that an operon under control by *AccR* contains a gene that could encode a protein showing extensive amino acid sequence homology to known methyl-accepting chemotaxis proteins (Farrand et al. 1996b; K. R. Piper and S. K. Farrand, *in preparation*). However, since AccA is sufficient for chemotaxis, it is clear that the product of this gene is not required for chemoattraction to the agrocinopines, at least when the system is expressed in the strains of *A. tumefaciens* that we tested.

The aberrant response to mannopine by strains harboring pDSRE10a is interesting. The insert in this clone confers transport of mannopine (Hong and Farrand 1994), and our recent sequence analysis indicates that it contains four genes that code for an ABC-type transport system (Farrand et al. 1996b; Oger et al., *in press*). Distal to these four genes is an open reading frame (ORF), *mcl*, the translation product of which shows homology to known Mcps (Oger et al., *in press*). However, the translated product of this ORF aligns only to the C-terminal portion of the related Mcps, suggesting that the protein is truncated at the N terminus. The C-terminal portion of the known Mcps forms the cytoplasmically located signal transduction domain (Stock and Surette 1996). The influence of the product of this ORF, if any, on chemotaxis is unknown. However, pRE10 and pYDH208 both code for the entire mannopine transporter but the inserts in these plasmids terminate before *mcl*. Neither of these clones confers the aberrant arcing response to mannopine (Fig. 4 and data not shown), but both of the plasmids mediate chemotaxis to mannopine (Table 2). The intact Ti plasmid pTi15955, however, does not confer the aberrant response to mannopine.

Strains harboring pAtK84b also exhibit chemotaxis to nopaline and to agrocinopines A+B, opines to which this plasmid confers catabolism. pAtK84b and pTiC58 share large regions of related sequences, including those corresponding to

the *noc* operon of pTiC58 (Clare et al. 1990). This suggests that the locus of pAtK84b that confers chemotaxis to nopaline may be similar to that of pTiC58. That pBC201, which contains the insert that shows strong relatedness to *noc* of pTiC58, elicited chemotaxis to nopaline (Table 2) supports this hypothesis. However, this clone did not allow NT1 to grow as well on this opine as did the entire plasmid, or pTHH6, the *noc* clone of pTiC58 (data not shown). This suggests that pBC201 lacks structural or regulatory genes required for wild-type expression of the *noc* phenotype. The determinant of pAtK84b specifying attraction to agrocinopines A+B may be different from *accA*. In contrast to the *noc* region of the two plasmids, there is, at best, only very weak relatedness between pAtK84b and the *acc* region of pTiC58 (Clare et al. 1990; Hayman and Farrand 1990). Nevertheless, pBC382, which contains that portion of pAtK84b that shows weak relatedness to *acc* of pTiC58, conferred chemotaxis to the agrocinopine opines (Table 2). This clone also conferred uptake of the agrocinopine opines (Fig. 4).

In pTiC58, the catabolism of agrocinopines A+B is governed by a repressor, *accR*, that also regulates conjugal transfer of the Ti plasmid in response to these two opines (Beck von Bodmann et al. 1992; Hayman et al. 1993). We observed that mutations in *accR*, which result in constitutive expression of *acc*, weakened chemotaxis to agrocinopines. It is possible that chemotaxis to the opines is regulated positively by *AccR*. More likely, derepression of *acc* due to the mutation in *accR* leads to increased channeling of the AccA-agrocinopine complex to the transport system rather than to the chemotaxis signal transducers. Strains NT1(pTiC58Δ*accR*) and NT1(pHK153) transport agrocinopines A+B at rates much faster than does the wild-type strain C58 (Hayman and Farrand 1988; Kim and Farrand 1997). Moreover, the *accR*⁺ and *accR*⁻ strains C58K438 and NT1K438, both with mutations in *accC*, no longer can transport the opines, and unlike their *accC*⁺ counterparts, do not differ in their chemotaxis to the agrocinopine opines (data not shown). This is consistent with our proposal and indicates that the mutant alleles of *accR* apparently are not directly responsible for the weakened chemotaxis to agrocinopines. Taken together, the data suggest that the three activities, chemotaxis to the opines, catabolism of the opines, and conjugal transfer of Ti plasmids, are coordinately regulated by the level of opines present in the environment.

We propose that chemotaxis to opines may be important in fostering the interaction between agrobacteria and their plant hosts. This is particularly intriguing for an organism like *A. radiobacter* strain K84. This bacterium does not itself induce galls but can utilize nopaline and agrocinopines A+B present in galls produced by the proper pathogenic agrobacteria. Strain K84 produces an antibiotic called agrocin 84 that inhibits the growth of nopaline/agrocinopine-type *A. tumefaciens* isolates (reviewed in Farrand 1991). The fact that strain K84 is chemoattracted by these very opines (Table 2) suggests that this behavioral activity may be the means by which this nontumorigenic bacterium recognizes and colonizes galls producing the opines it is equipped to catabolize.

Overall, in that opines are specific chemoattractants, we suggest that these novel tumor metabolites can play an important role in determining the distribution of agrobacteria in the tumorsphere. Moreover, our data lend further support to the model proposed by Tempé et al. (1979) that opines are the

Table 4. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype, phenotype, or characteristics ^a	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al. 1989
S17-1	Pro ⁺ Res ⁺ Mod ⁺ <i>recA</i> ; integrated RP4 ⁺ Tc ^r ::Mu-Kan::Tn7, Mob ⁺ ; Sm ^r	Simon et al. 1983
<i>Agrobacterium radiobacter</i>		
K84	Nop ⁺ , Acp ⁺ (pAgK84, pAtK84b)	Our collection
<i>A. tumefaciens</i>		
C58	Nop ⁺ , Acp ⁺ (pTiC58, pAtC58)	Our collection
NT1	C58 cured of pTiC58	Watson et al. 1975
SA122	15955 cured of pTi15955	Our collection
15955	Oct ⁺ , AGA ⁺ , AGR ⁺ , MOA ⁺ , MOP ⁺ (pTi15955)	Our collection
R10	Oct ⁺ AGA ⁺ , AGR ⁺ , MOA ⁺ , MOP ⁺ (pTiR10)	Our collection
NT1(pTiC58 Δ <i>accR</i>)	Nop ⁺ , Acp ^c	Beck von Bodman et al. 1992
NT1(pTi15955)	Oct ⁺ , AGA ⁺ , AGR ⁺ , MOA ⁺ , MOP ⁺	Our collection
SA122(pTi15955)	Oct ⁺ , AGA ⁺ , AGR ⁺ , MOA ⁺ , MOP ⁺	Our collection
NT1(pTiR10)	Oct ⁺ , AGA ⁺ , AGR ⁺ , MOA ⁺ , MOP ⁺	Our collection
NT1(pAtK84b)	Nop ⁺ , Acp ⁺	Our collection
NT1(pAgK84)	Agrocin 84-producing strain	Our collection
C58K11	pTiC58 <i>accG</i> ::Tn3HoHo1 11; Cb ^r	Kim and Farrand 1997
C58K438	pTiC58 <i>accC</i> ::Tn3HoHo1 438; Cb ^r	Kim and Farrand 1997
C58KF	pTiC58 <i>accF</i> :: <i>lacZ</i>	Kim and Farrand 1997
NT1K438	pTiC58 Δ <i>accR</i> <i>accC</i> ::Tn3HoHo1 438; Cb ^r	This study
KYC8	R10 pTiR10 <i>ooxB</i> ::Tn5- <i>gusA7</i> ; Km ^r	Cho et al. 1996
KYC11	R10 pTiR10 <i>occQ</i> ::Tn5- <i>gusA7</i> ; Km ^r	Cho et al. 1996
KYC16	R10 pTiR10 <i>ooxA</i> ::Tn5- <i>gusA7</i> ; Km ^r	Cho et al. 1996
KYC17	R10 pTiR10 <i>ooxM</i> ::Tn5- <i>gusA7</i> ; Km ^r	Cho et al. 1996
SA122 (pTi R10-8)	pTiR10 <i>ooxB</i> ::Tn5- <i>gusA7</i> ; Km ^r	This study
SA122 (pTi R10-11)	pTiR10 <i>occQ</i> ::Tn5- <i>gusA7</i> ; Km ^r	This study
SA122 (pTi R10-16)	pTiR10 <i>ooxA</i> ::Tn5- <i>gusA7</i> ; Km ^r	This study
SA122 (pTi R10-17)	pTiR10 <i>ooxM</i> ::Tn5- <i>gusA7</i> ; Km ^r	This study
NT1(pBC201)	pVK102::HindIII partial of pAtK84b (coordinates 0-20.7); Tc ^r	This study
NT1(pBC382)	pVK102::HindIII partial of pAtK84b (coordinates 144.47-172.85); Tc ^r	This study
NT1(pRE10)	pRK415K::EcoRI-HindIII from pYDH208; Km ^r	Hong and Farrand 1994
NT1(pDSRE10a)	pDSK519::EcoRI fragment 10 from pTIR10; Km ^r	Oger et al., <i>in press</i>
NT1(pDSRE10b)	pDSK519::EcoRI fragment 10 from pTIR10 (other orientation); Km ^r	Oger et al., <i>in press</i>
Plasmids		
pYDH23	pCP13::HindIII partial of pTi15955; Tc ^r	Dessaux et al. 1987
pYDH208	pCP13::HindIII partial of pTi15955; AGR ⁺ , MOP ⁺ , Tc ^r	Hong and Farrand 1994
pYDH304	pCP13::HindIII partial of pTi15955; AGA ⁺ , MOA ⁺ , MOP ⁺ , Tc ^r	Dessaux et al. 1987
pYDH501	pCP13::HindIII partial of pTi15955; Oct ⁺ , Tc ^r	Dessaux et al. 1987
pSaB4	pSa4 Δ EH::BamHI fragment 4 of pTi15955; AGR ⁺ , Km ^r	Kim and Farrand 1996
pTHH6	pCP13::HindIII partial of pTiC58; Nop ⁺ Tc ^r	Hayman 1989
pTHH17	pCP13::HindIII partial of pTiC58; Tc ^r	Hayman 1989
pTHH23	pCP13::HindIII partial of pTiC58; Tc ^r	Hayman 1989
pTHE16	pCP13::HindIII partial of pTiC58; Tc ^r	Hayman 1989
pTHSS9	pSa152::SmaI-9 of pTiC58; Km ^r	Hayman 1989
pTHH2	pVK102::HindIII partial of pTiC58; Tc ^r	Hayman 1989
pTHH7	pVK102::HindIII partial of pTiC58; Tc ^r	Hayman 1989
pTHH32	pVK102::HindIII partial of pTiC58; Tc ^r	Hayman and Farrand 1988
pTHSB5	pSa152::BamH I-5 of pTiC58; Km ^r	Hayman and Farrand 1988
pTHB112	pCP13::BamHI partial of pTiC58; Acp ⁺ , Tc ^r	Hayman and Farrand 1988
pTHB403	pTHB112::Tn3HoHo1 403; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB406	pTHB112::Tn3HoHo1 406; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB408	pTHB112::Tn3HoHo1 408; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB409	pTHB112::Tn3HoHo1 409; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB418	pTHB112::Tn3HoHo1 418; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB431	pTHB112::Tn3HoHo1 431; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB464	pTHB112::Tn3HoHo1 464; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB469	pTHB112::Tn3HoHo1 469; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB476	pTHB112::Tn3HoHo1 476; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pTHB478	pTHB112::Tn3HoHo1 478; Acp ⁺ , Tc ^r , Cb ^r	Hayman and Farrand 1988
pDS3.1	pDSK519::SalI-3.1 of pTiC58; Km ^r	This study
pBC201	pVK102::HindIII partial of pAtK84b (coordinates 0-20.7); Tc ^r	Clare et al. 1990
pBC382	pVK102::HindIII partial of pAtK84b (coordinates 144.47-172.85); Tc ^r	Clare et al. 1990
pHK11	pTHB112::Tn3HoHo1 11; Tc ^r , Cb ^r	Kim and Farrand 1997
pHK23	pTHB112::Tn3HoHo1 23; Acp ⁺ , Tc ^r , Cb ^r	Kim and Farrand 1997
pHK76	pTHB112::Tn3HoHo1 76; Acp ⁺ , Tc ^r , Cb ^r	Kim and Farrand 1997
pHK115	pTHB112::Tn3HoHo1 115; Acp ⁺ , Tc ^r , Cb ^r	Kim and Farrand 1997
pHK153	pTHB112::Tn3HoHo1 153; Acp ⁺ , Tc ^r , Cb ^r	Kim and Farrand 1997
pHK438	pTHB112::Tn3HoHo1 438; Tc ^r , Cb ^r	Kim and Farrand 1997
pHKF	pTHB112(<i>accF</i> :: <i>lacZ</i>); Tc ^r	Kim and Farrand 1997

^a Abbreviations: Amp^r, ampicillin resistance; Cb^r, cabenecillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Acp⁺, agrocinopines A+B utilization; Acp⁻, unable to utilize agrocinopines A+B; Acp^c, constitutive utilization of agrocinopines A+B; Nop⁺, nopaline utilization; Oct⁺, octopine utilization; Oct⁻, unable to utilize octopine; AGA⁺, agropinic acid utilization; AGR⁺, agropine utilization; MOA⁺, mannopinic acid utilization; MOP⁺ mannopine utilization.

key chemical mediators that establish the *Agrobacterium*-plant interaction.

MATERIALS AND METHODS

Bacterial strains and plasmids.

All bacterial strains and plasmids used in these studies are described in Table 4.

Media and chemicals.

Agrobacterium strains were grown in Luria-Bertani (LB, Gibco BRL, Gaithersburg, MD) medium, nutrient agar (NA, Difco Laboratories, Detroit, MI) medium, or AB minimal medium (Chilton et al. 1974) with 0.25% mannitol. Nutrient broth (1×) plates with 0.2% agar were used to enrich for motile agrobacteria. For the classic swarm plate assay, 5 ml of AB minimal medium with 0.25% noble agar and a sugar at 0.025% or an opine at 0.1 mM or at 1 mM was used. Plates containing 5 ml of AB minimal medium with 0.2% agar and 0.025% mannitol were used for directional swarm plate assays. *E. coli* strains were cultured in LB medium. Antibiotics were used at the following concentrations: For *E. coli*, tetracycline (Tc), 10 µg/ml; ampicillin (Amp), 100 µg/ml; and kanamycin (Km), 50 µg/ml. For *Agrobacterium* strains: kanamycin, 50 or 100 µg/ml; tetracycline, 2 µg/ml; and carbenicillin (Cb), 100 or 200 µg/ml. A mixture of agrocino-pines A+B was purified from tomato crown galls induced by *A. tumefaciens* strain T37 as described by Ryder et al. (1984). Nopaline, mannopine, and octopine were purchased from Sigma Chemical Co. (St. Louis, MO).

Genetic manipulations.

E. coli strains were transformed by the CaCl₂ procedure of Sambrook et al. (1989). *A. tumefaciens* strains were electroporated according to the procedure of Cangelosi et al. (1991). Ti plasmids were isolated as described by Hayman and Farrand (1990). Recombinant plasmids based on IncP1 vectors or on IncQ vectors were mobilized into *A. tumefaciens* by a biparental method with *E. coli* strain S17-1 (Farrand et al. 1996a). This strain harbors a derivative of RP4 integrated into its chromosome (Simon et al. 1983). Tn3HoHo1 insertion 438 in pHK438 was marker-exchanged into pTiC58 and pTiC58Δ*accR* as previously described (Garfinkel et al. 1981). The marker-exchange mutations were confirmed by appropriate phenotypes and by restriction endonuclease analysis of purified Ti plasmids.

Uptake of agrocino-pines A+B.

Strains were tested for their ability to remove opines from the medium by the procedure described by Kim and Farrand (1997). Cells were grown in AB minimal medium with mannitol to an OD₆₀₀ of 0.3, collected by centrifugation, washed with 0.9% NaCl, and resuspended in the same volume of AB minimal medium without any carbon source. Thirty-microliters of AB minimal medium containing agrocino-pines A+B at a concentration of 3 mM was inoculated with 1 µl of the cell suspension. Cultures were incubated at 28°C, and 10-µl samples were removed at 0 and at 48 h. The cells were removed from the samples by centrifugation, and the supernatants were analyzed for the presence of agrocino-pines A+B by high voltage paper electrophoresis (HVPE) as described previously (Hayman and Farrand 1988).

Chemotaxis assays.

The classic swarm plate assay was conducted essentially as described by Adler (Adler 1966; Armstrong et al. 1967). In this assay, tested sugars or opines were added to the medium as described in Materials and Methods. *Agrobacterium* strains to be tested were first passed through several nutrient broth motility plates to enrich for motile bacteria. Bacterial cells at the edge of the swarm zone were taken with an inoculating loop and were streaked on AB minimal mannitol (ABM) agar medium containing appropriate antibiotics. A single colony was picked and was streaked on a second plate of ABM agar medium. After colonies formed on the plates, cells, taken with a toothpick, were inoculated onto the center of a swarm assay plate. The plates were incubated at 28°C until distinct rings of bacterial growth were formed. A modified directional swarm assay based on that described by Hawes et al. (1988) also was used. Bacteria passed through several nutrient broth motility plates and streaked on ABM plates as described above were inoculated onto the center of the assay plates with a toothpick. A 10-µl volume of a 5 mM stock solution of a given opine or of sucrose was spotted to the edge of the plates and the plates were incubated overnight or longer at 28°C before the assays were scored.

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