JOURNAL OF BACTERIOLOGY, Nov. 1984, p. 564–568
0021-9193/84/110564-05$02.00/0
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Vol. 160, No. 2

Physical and Functional Map of an *Agrobacterium tumefaciens* Tumor-Inducing Plasmid That Confers a Narrow Host Range

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Received 14 May 1984/Accepted 30 July 1984

Agrobacterium tumefaciens **Ag162 induces crown gall disease on an unusually narrow range of host plants. The 231-kilobase Ti plasmid which has been shown to determine host range, was subcloned into the vector pVCK102. By comparing overlaps of cloned insets, maps were constructed for the restriction endonucleases *SalI*, *XhoI*, *EcoRI*, and *KpnI*. Plasmid incompatibility, octopine catabolism, and at least six virulence genes were localized. Plasmid incompatibility between pTiAg162 and the wide host range plasmid pTiA6 consists of two components: mutual incompatibility and the apparent ability of pTiA6 to block RK2 replication if the pTiAg162 incompatibility locus is linked to the vector pVK102. The octopine catabolism locus maps within the 30 kilobases of DNA separating the two T-DNA regions of pTiAg162. Complementation of avirulent *vir* mutants of pTiA6 with clones of pTiAg162 DNA did not confer the host range of pTiAg162 but rather restored the wide host range of pTiA6. One potentially important difference between pTiA6 and pTiAg162 is that pTiAg162 T-DNA regions are widely separated.**

Crown galls are plant tumors incited by the pathogen *Agrobacterium tumefaciens* (see references 1, 4, and 18 for reviews). The nuclear DNA of transformed plant cells contains specific DNA sequences (T-DNA) transferred from *A. tumefaciens* to the host plant. In *A. tumefaciens* cells, the T-DNA represents a small portion of the tumor-inducing (Ti) plasmid. Other regions of the Ti plasmid code for replication (17), opine catabolism (5, 13, 19), and functions required for tumor induction (*vir* genes) (11, 12). The T-DNA genes expressed in the transformed plant cell (7, 23) include the oncogenes as well as genes coding for the synthesis of tumor-specific compounds called opines (20) which support the growth of *A. tumefaciens*.

Most *A. tumefaciens* strains can induce tumors on most dicotyledonous plants as well as on some gymnosperms (2). However, strains have been isolated which induce tumors on relatively few plant species. The Ti plasmid has been shown to be largely responsible for host range differences among *A. tumefaciens* strains (14, 22). To study the molecular basis of host range differences, we determined the physical map as well as a partial genetic map of a Ti plasmid coding for a limited host range, pTiAg162, and compared these maps with those of the relatively well-characterized plasmid pTiA6 (18).

MATERIALS AND METHODS

Escherichia coli HB101 was the host for all cosmid clones. *A. tumefaciens* strains A348 and A856 have been described previously (13). The procedures for cosmid cloning and restriction enzyme mapping and the determinations of octopine catabolism and incompatibility were similar to methods previously described (9, 13). Tn*5* mutagenesis of cosmid clones, subsequent marker exchanges into the Ti plasmid, and virulence assays were done by the method of Garfinkel et al. (6). *A. tumefaciens* strains with *vir* lesions have been described by Klee et al. (12) and Iyer et al. (10).

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RESULTS

Cosmid clone banks of pTiAg162 DNA. Two cosmid clone banks of pTiAg162 DNA were constructed and analyzed. The cloning vector pVK102 can be maintained in *A. tumefaciens* as well as in *E. coli* (13). One bank, represented by plasmids with "300" numbers in Table 1, was constructed by partially digesting pTiAg162 with *SalI* and then cloning into the *SalI* site of pVCK102. These plasmids code for resistance to kanamycin but not to tetracycline since inserts cloned into the *SalI* site of pVCK102 inactivate the tetracycline resistance gene. Plasmids with "400" numbers in Table 2 made up the second bank. The plasmid pTiAg162 was partially digested with *XhoI*, and the fragments were then cloned into the *XhoI* site of the kanamycin resistance gene.

Restriction endonuclease maps of pTiAg162. Restriction endonuclease maps of pTiAg162 DNA were deduced by comparing clones with overlapping inserts (3, 13). Restriction fragments shared by two plasmids whose cloned inserts overlap were mapped to the region the two plasmids have in common. The clones listed in Tables 1 and 2 were sufficient to order almost all of the fragments larger than 800 base pairs. Additional map data were acquired by analysis of selected double digests and fine-scale mapping of transposon insertions (see below). Figure 1 shows the relative positions of the *SalI*, *XhoI*, *EcoRI*, and *KpnI* sites of pTiAg162. The four maps were aligned by *SalI*-*KpnI*, *XhoI*-*KpnI*, and *EcoRI*-*KpnI* double digests of selected clones which "walk around" the pTiAg162 plasmid. The pTiAg162 genome is slightly larger than 231 kilobases (kb).

The *XhoI* sites at 4.5 and 220.2 kb and the *SalI* site at 213.3 kb in pTiAg162 (Fig. 1) were not recognized by these enzymes. However, these same sites were recognized when clone DNA was isolated from *E. coli* HB101. Southern blots of whole Ti plasmid digested with either *SalI* or *XhoI* probed with cloned DNAs did not reveal even partial cutting at these sites. When cloned DNA was isolated from *A. tumefaciens* A136, these sites were only partially digested by excessive amounts of enzyme even though all other sites appeared completely cut. The pTiAg162 DNA preparations used for

TABLE 1. *Sal*I clone bank of pTiAg162

Cosmid clone	Coordinates (kb)	Cosmid clone	Coordinates (kb)
pVCK		pVCK	
300	184.4–208.55	333	92.0–113.7
301	172.35–206.7	334	96.8–120.75
302	184.4–204.75	335	100.3–120.75
303	172.35–190.85	336	113.7–134.2
304	179.05–202.05	338	113.7–134.2
305	190.85–206.7	339	131.55–149.65
306	206.7–230.6	340	120.75–145.3
307	208.55–230.6	341	134.2–157.7
309	208.55–230.6	342	57.35–76.20
310	223.1–231.25, 0–9.65	350	210.5–231.25, 0–0.35
311	224–231.25, 0–20.45	353	149.65–172.35
312	0.85–26.1	355	204.75–224.0
313	37.2–64.8	366	113.7–138.75
314	29.2–52.25	368	7.3–29.2
316	29.2–52.25	369	17.9–37.2
317	17.9–37.2	372	145.3–161.95
318	37.2–58.95	374	92.0–113.7
319	29.2–57.35	376	37.2–58.95
320	37.2–57.35	377	172.35–190.85
321	28.2–46.0	378	76.2–99.35
322	29.2–46.0	379	157.7–178.55
324	23.1–52.25	380	100.3–120.75
325	76.2–92.0	394	161.95–184.4
326	76.2–99.35	395	175.05–196.15
328	76.2–96.8	396	204.75–230.6
329	64.8–87.5	397	37.2–57.35
331	64.8–85.5	398	120.75–149.65
332	92.0–113.7	399	96.8–120.75

cloning were independently isolated for each bank; *Xho*I or *Sal*I restriction patterns showed no polymorphism. No clones in either bank contained the *Xho*I or *Sal*I site of the vector directly fused to any of the three sites in question. Thus, the sites were not recognized when the *A. tumefaciens* DNA was partially digested before ligation. An *A. tumefaciens* DNA modification system may be involved, but we have no direct data to support this hypothesis.

Octopine catabolism loci of pTiAg162. Since pVCK102 and pVCK102 with a cloned insert are stably maintained in *A. tumefaciens* cells, pTiAg162 genetic loci can be mapped by examining the expression of cloned genes in *A. tumefaciens* (13). Cosmid clones representing specific regions of pTiAg162 were mated into strain A114, which contains no Ti plasmid and thus does not utilize octopine. Strain A114 containing pVCK312 can utilize octopine (13). When cosmids with cloned inserts overlapping the one in pVCK312 were tested, pVCK368, -414, -416, -423, -427, and -428 all coded for octopine catabolism. All of these plasmids contained the region between coordinates 16.0 and 24.8. Since strain A114 does not normally contain the octopine permease and the octopine catabolic enzyme(s), the genes for these proteins as well as their genetic control elements must lie between coordinates 16.0 and 24.8. pVCK425 extends from coordinate 0 to 19.6 and does not code for octopine catabolism; pVCK429 encompasses the region from coordinate 19.6 to 40.8 and also does not code for octopine catabolism. Thus, one locus essential for octopine catabolism spans the physical coordinate at 19.6 (Fig. 2).

Plasmid incompatibility locus of pTiAg162. pTiAg162 has previously been shown to be incompatible with the Ti plasmid pTiA6 (13). The incompatibility locus of pTiAg162 was mapped physically by identifying cosmid clones which excluded pTiA6 from *A. tumefaciens* A348. Introduction of pVCK326, -332, or -334 into strain A348 and selection for kanamycin resistance resulted in the loss of pTiA6. Tetracycline resistance is not a good selectable marker in strain A348, so the 400 series of cosmids were not tested. Only those three cosmids expressed incompatibility; 300 series of cosmids containing other regions of pTiAg162 can coexist stably with pTiA6. pVK328 extends from coordinates 76.2 to 96.8; pVK326 extends from coordinates 76.2 to 99.35. Since pVK326 but not pVK328 was incompatible with pTiA6, the DNA from coordinates 96.8 to 99.35 must contain at least part of the genetic locus concerned with incompatibility. This conclusion is supported by several other observations; pVK334 exerted incompatibility and extends from coordinates 96.8 to 120.75, whereas pVCK335 was compatible with pTiA6 and contains the DNA from coordinate 100.3 to 120.75. Furthermore, the region between coordinates 96.8 and 99.35 was the only region shared by the cloned inserts of pVCK326 and pVCK334. Since the origin of replication in pTiA6 (17) and most other plasmids is closely linked to plasmid incompatibility determinants, we suggest that the origin of pTiAg162 replication is near or in the 2.55-kb region between coordinates 96.8 and 99.35.

The plasmids pVCK326, -332, and -334 were transferred at unusually low frequencies into strain A348 but not into strain A114. The transfer frequency was measured as the ratio of kanamycin-resistant *A. tumefaciens* cells to the total number of cells after a triparental mating (13), using *E. coli* HB101 with the specified cosmid clone, *E. coli* HB101 with the

TABLE 2. *Xho*I clone bank of pTiAg162

Cosmid clone	Coordinates (kb)	Cosmid clone	Coordinates (kb)
pVCK		pVCK	
400	183.55–199.2	437	73.95–95.5
401	177.75–199.2	438	73.95–100.5
402	176.65–199.2	439	84.5–105.5
403	177.75–201.7	440	84.5–100.5
404	177.75–201.7	441	71.45–96.5
405	184.85–213.2	442	100.5–118.55
406	184.85–213.2	443	105.5–128.75
407	205.8–223.7	444	114.2–128.75
408	196.05–223.7	445	100.5–118.55
409	201.7–229.8	447	95.5–120.05
410	196.5–223.7	448	105.5–135.25
411	131.05–156.55	449	114.2–140.05
412	205.8–226.0	451	137.55–156.55
413	201.7–228.5	452	135.25–156.55
414	0–24.8	453	135.25–156.55
415	0-24.8	454	151.25–168.55
416	226–231.25, 0–24.8	456	141.75–168.55
417	226–231.25, 0–24.8	459	156.55–177.75
418	216.7–231.25, 0-16.0	460	156.55–176.65
419	223.7–231.25, 0–16.0	467	205.8–231.25
420	223.7–231.25, 0–14.7	468	40.8–71.45
421	226–231.25, 0–16.0	469	67.1–91.5
422	226–231.25, 0–14.7	470	168.55–184.85
423	0–29.4	471	114.2–133.75
424	226–231.25, 0–19.6	472	120.05–140.05
425	0–19.6	474	71.45–93.2
426	20.7–40.8	475	151.25–196.05
427	16.0–40.8	476	100.5–114.2
428	14.7–40.8	477	168.55–193.55
429	19.6–40.8	478	199.1–226
430	29.4–48.5	479	177.75–201.7
431	34.0–60.9	480	0–14.7
432	40.8–67.1	481	40.8–64.6
434	42.7–60.9	483	114.2–133.75
435	42.7–67.1		

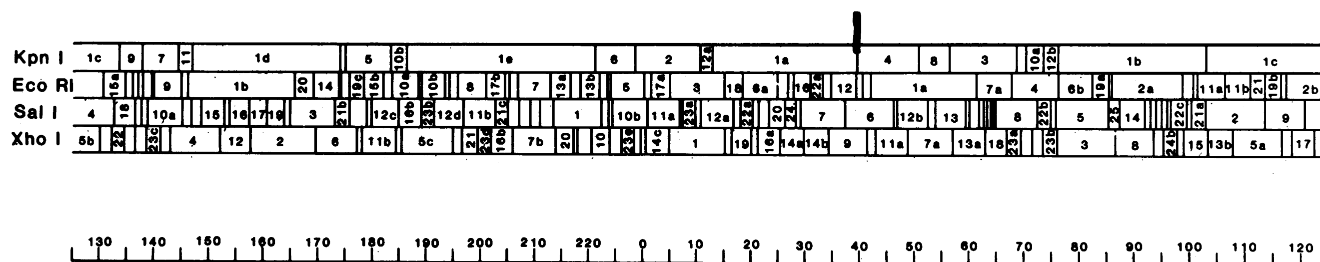

FIG. 1. Restriction map of pTi-Ag162. *Kpn*I, *Eco*RI, *Sal*I, and *Xho*I restriction sites are designated for the 231-kb limited host range plasmid. Restriction fragments are designated according to their relative size. Below the restriction map is shown the coordinates (kb) which correspond to the clones listed in Tables 1 and 2.

helper plasmid pRK2013, and an *A. tumefaciens* strain containing pTiA6 (A348), pTiAg162 (A856), or no Ti plasmid (A114). The frequency of transfer for pVCK102, -326, -332, and -334 into strain A114 ranged from 10^{-2} to 10^{-3}. The same frequency was observed for pVCK102 and all other clones tested into strain A114 or A348. The plasmids coding for incompatibility transferred into strain A348 at frequencies ranging from 10^{-6} to 10^{-8}. Since Ti plasmids do not code for surface exclusion (8), these cosmids were presumably transferred into strains A348 and A114 at similar frequencies but became established in strain A348 at a rate some 10^{-4} lower. This is remarkable because the cosmids contain an independent RK2 replicon that is compatible with pTiA6. It appears that the 2.55 kb of DNA containing the incompatibility locus of pTiAg162 in these cosmids enables the resident pTiA6 of strain A348 to interfere with the establishment of the RK2 replicon.

Interestingly, pTiAg162 did not exert this effect on pVCK326, -332, or -334. Moreover, cosmid clones of pTiA6 which contain the incompatibility locus of pTiA6 (including pVCK211) had conjugal transfer frequencies of 10^{-2} to 10^{-3} into either strain A348 containing pTiA6 or strain A856 containing pTiAg162. Plasmid incompatibility appears to be mutual between pTiA6 and pTiAg162, but pTiA6 can affect the establishment of some pTiAg162 clones in *A. tumefaciens* without the converse being true.

Virulence genes of pTiAg162. Virulence genes are the genetic loci of *A. tumefaciens* required for plant tumorigenesis that are not located in the T-DNA (18). At least five *vir* loci have been mapped by transposon mutagenesis in the wide host range Ti plasmid pTiA6 (12). One of these, *virB*, is conserved among many Ti plasmids as determined by DNA homology and appears to have more than one gene required for virulence as indicated by the locus size and complementation behavior (10). We localized the *vir* loci in pTiAg162 by three methods: (i) mapped *vir* mutants of pTiA6 were complemented in *trans* by cosmid clones of pTiAg162; (ii) cosmid clones of pTiAg162 DNA were mutagenized by transposon Tn*5* insertions, and these clones were then tested for their ability to complement *vir* mutants of pTiA6; and (iii) the Tn*5* insertions into cosmid clones were recombined into the pTiAg162 genome by a marker exchange technique (6), and these mutants were tested for virulence on *Nicotiana glauca* plants.

Transposon insertions near coordinate 199 or 202 of pTiAg162 abolished virulence (Fig. 3). These mutations were introduced into the pTiAg162 genome via intermediate cosmid clone pVCK405 or pVCK408 with Tn*5* insertions at these positions. Tn*5* insertions at other sites did not affect virulence; cosmid clone pVCK405 bearing silent insertions restored virulence to the avirulent Tn*3* mutants of pTiA6 in *A. tumefaciens* strains A2005, A2019, A2002, A2003, A2046, and A2001, which span an ca 9-kb region defined as the *virB* region (12).

Two Tn*5* insertions near coordinate 199 and four near 202 resulted in cosmid clones that no longer complemented any of the *virB* lesions in the above strains. For the pTiAg162 *vir* gene(s), a single Tn*5* insertion abolished the ability to complement six separate *virB* mutations in pTiA6. This suggests that the region of pTiAg162 corresponding to the

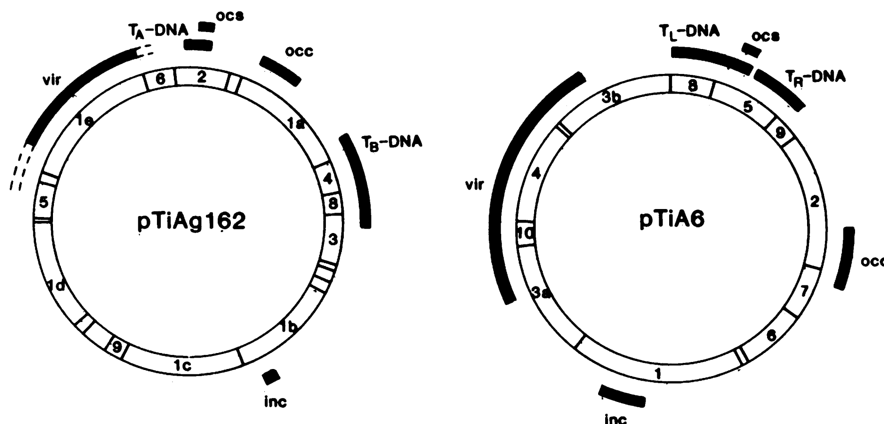

FIG. 2. Comparison of limited and wide host range octopine-type Ti-plasmids. Circular restriction and functional maps are shown for pTiAg162 and pTiA6. *Kpn*I restriction sites are designated for both plasmids. Also shown are the locations of the regions corresponding to *vir* genes, T-DNAs, octopine synthase (OCS), octopine catabolism (OCC), and plasmid incompatibility (INC).

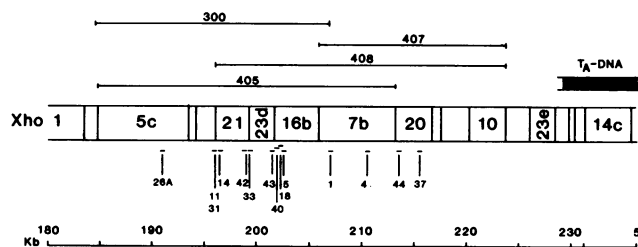

FIG. 3. Analysis of virulence region of pTiAg162. A *Xho*I restriction map is shown for the region spanning the *vir* loci of pTiAg162. Coordinates (kb) are shown below this map and correspond to the restriction map in Fig. 1. Also shown are the regions encompassed by cosmid clones pVCK 405, -407, -408, and -300. The locations of Tn*5* insertion mutants described in the text are also shown. Mutants were assayed on *N. glauca* plants. The location of the T_A-DNA region is also shown for reference.

virB region of pTiA6 may consist of a single transcriptional unit. The clone pVCK407 lacked the DNA from coordinate 199 to 202, yet it complemented the *vir* lesion in strain A2000. Furthermore, a Tn*5* insertion in pVCK407 near coordinate 207 no longer complemented A2000. One explanation for the observation that pVK407 can complement A2000 without the genetic loci identified by insertions at coordinates 199 and 202 is that transcriptional readthrough from the cloning vector resulted in expression of this putative *virB* gene. The *virB* region of pTiAg162 clearly extends from coordinate 199 past coordinate 206 since pVCK300 (coordinates 184.4 to 208.55) complements *virB* lesions but pVCK301 (coordinates 172.35 to 206.7) does not. Other transposon insertions into pTiAg162 created avirulent strains, but these insertions did not affect the ability of the intermediate cosmid clone to complement *virB* lesions. Thus, the *vir* loci identified at coordinates 191, 196, 210.5, 213.4, and 216.5 (Fig. 3) may correspond to other virulence genes in pTiA6. Since cosmid clones with pTiAg162 DNA corresponding to coordinates 206 to 224 restored virulence to the *virD* mutant strain A2010 and the *virE* mutant strain A2011 (Table 3), we conclude that the pTiAg162 *vir* loci equivalent to *virD* and *virE* of pTiA6 reside within this region.

Complementation of these *vir* mutants of pTiA6 was observed on *N. glauca*, *Nicotiana tabacum* var. *xanthi*, and *Kalanchoe daigremontiana*. All three are natural hosts for *A. tumefaciens* strains with pTiA6; however, the latter two plant species are normally resistant to infection by *A. tumefaciens* strains with pTiAg162. Since complementation

TABLE 3. Complementation of *A. tumefaciens* plasmids

Strain	Wide host range plasmid pTiA6 mutation	Complemented by[a]:		
		pVCK405	pVCK407	pVCK408
A2005	*virB*	+	−	+
A2019	*virB*	+	−	+
A2002	*virB*	+	−	+
A2003	*virB*	+	−	+
A2046	*virB*	+	−	+
A2000	*virB*	+	+	+
A2010	*virD*	−	+	+
A2011	*virE*	−	+	+

[a] pVCK405, pVCK407, and pVCK408 are cosmid subclones of the limited host range plasmid pTiAg162.

with *vir* genes of pTiAg162 restored a wide host range, the *virB*, *-D*, and *-E* genes of pTiAg162 are not responsible for the limited host range coded by pTiAg162.

Thus, we identified and mapped the loci of pTiAg162 which appear equivalent to the *virB*, *-D*, and *-E* loci of pTiA6 by the functional test of complementation. Like pTiA6, these loci are physically located within one region of the Ti plasmid which is separated from the T-DNA regions of pTiAg162.

Position of T-DNA sequences in the pTiAg162 genome. Two distinct T-DNAs of pTiAg162 have previously been identified by their presence in tumor lines of grapevine and of *N. glauca* (manuscript in preparation). T_A-DNA falls within the 230- and 6-kb coordinates of the pTiAg162 map and T_B-DNA extends from coordinates 38 to 55. Figure 2 indicates the relative spatial separation of these two T-DNAs, which are transferred and stably maintained as part of the host plant genome. The more than 30 kb of DNA separating the two T-DNAs contains octopine catabolism genes which are expressed in the bacterium but have no direct role in the expression of virulence. DNA homology was not detected between the "satellite" T-DNA of pTiA6 and pTiAg162 DNA (data not shown). The lack of any homology to the region of pTiA6 DNA coding for agropine synthesis is consistent with the observation that tumors induced by strains with pTiAg162 did not synthesize agropine.

DISCUSSION

As well as constructing a physical map of the limited host range plasmid pTiAg162 for four different restriction enzymes, we mapped genetic loci associated with (i) octopine catabolism; (ii) plasmid incompatibility; (iii) virulence genes functionally equivalent to *virB*, *-D*, and *-E* of pTiA6; and (iv) the two T-DNAs of pTiAg162.

The functional organization of plasmids pTiC58 and pTiA6 (see references 1 and 18 for reviews) taken together with these data for pTiAg162 reveal no hard and fast rules for the organization of genetic information in Ti plasmids. Although the *virB* region contains tightly linked genes which share some elements of common control, other *vir* loci appear nearby but are separated by DNA not required for tumorigenesis in the laboratory. It also appears that genes coding for catabolism of an opine are linked, but catabolic operons for different opines may be widely separated (e.g., agrocinopine and nopaline catabolism in pTiC58). To date, all Ti plasmids examined contain substantial amounts of DNA sequences for which there are no known functions.

The evolutionary divergence of wide host range pTiA6 and narrow host range pTiAg162 is obvious from DNA homology experiments (15, 21). These two Ti plasmids share a number of similarities but differ in genetic organization. pTiA6 and other octopine wide host range Ti plasmids which share DNA homology were all isolated from biotype 1 strains of *A. tumefaciens*, whereas Ti plasmids which share high DNA homology with pTiAg162 were all isolated first from biotype 3 strains of *A. tumefaciens*. However, Ti plasmids of wide host range and limited host range strains belong to the same incompatibility group, and detectable DNA homology to pTiAg162 can be visualized in regions spread around the pTiA6 genome (15, 21). The curious one-way incompatibility exerted by pTiA6 may reflect a mechanism by which Ti plasmids aggressively compete with incoming Ti plasmids as opposed to a passive incompatibility situation in which plasmids segregate to different daughter cells over time. It may also be related to the observation of Hooykaas et al. (8) that selection for an incoming plasmid revealed recombina-

tion between the incoming and resident plasmid rather than loss of the resident Ti plasmid.

The similarities between pTiA6 and pTiAg162 include the virulence genes. All *vir* mutants of pTiA6 tested could be complemented by clones of pTiAg162 DNA. Since a wide host range was restored to *vir* mutants of pTiA6 by cloned *vir* genes of pTiAg162, these loci do not critically determine host range. The *virA* or *-C* (or yet unidentified) locus may be involved in host range expression, but complementation of pTiAg162 with cloned *vir* regions of pTiA6 did not extend host range (16). Indeed, it is not the *vir* region of pTiA6 but the T-DNA region of pTiA6 that extended the host range of strains with pTiAg162 (16). The most striking molecular difference between pTiA6 and pTiAg162 is the spatial separation of the two T-DNA regions in pTiAg162. pTiA6 also has two T-DNAs but these are very closely linked in contrast to the widely separated T-DNA regions of pTiAg162.

ACKNOWLEDGMENTS

We thank Paul Ebert, Alison Rattray, and William Widner for isolating transposon insertions in the *vir* region.

This research was supported in part by grant PCM-8104310 from the National Science Foundation.

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