The Conjugal Intermediate of Plasmid RSF1010 Inhibits Agrobacterium tumefaciens Virulence and VirB-Dependent Export of VirE2

LISA E. STAHL, AMY JACOBS, AND ANDREW N. BINNS*
Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania

Received 13 February 1998/Accepted 30 May 1998

Agrobacterium tumefaciens causes crown gall disease by transferring oncogenic, single-stranded DNA (T strand), covalently attached to the VirD2 protein, across the bacterial envelope into plant cells where its expression results in tumor formation. The single-stranded DNA binding protein VirE2 is also transferred into the plant cell, though the location at which VirE2 interacts with the T strand is still under investigation. The movement of the transferred DNA and VirE2 from A. tumefaciens to the plant cell depends on the membrane-localized VirB and VirD4 proteins. Further, the movement of the IncQ broad-host-range plasmid RSF1010 between Agrobacterium strains or from Agrobacterium to plants also requires the virB-encoded transfer system.

Here we demonstrate that the capacity to form a conjugal intermediate of RSF1010 is necessary for this inhibition, suggesting that the transferred form of the plasmid competes with the VirD2-T strand and/or VirE2 for a common export site.

The soil phytopathogen Agrobacterium tumefaciens transforms plant cells by transporting DNA, mobilized from a tumor-inducing (Ti) plasmid located in the virulent bacterium, into the plant cell nucleus. Expression of this transferred DNA (T-DNA) leads to the formation of crown gall tumors on most dicotyledonous plants (for a review, see reference 25). The Ti plasmid also contains the virulence (vir) region, which provides several gene products that mediate transformation. The T-DNA from the Ti plasmid undergoes site-specific nicking at the 23-bp border repeats by VirD2, and a single-stranded DNA intermediate, covalently bound at its 5’ end to VirD2, is formed (for reviews, see references 25, 38, and 51). A single-stranded DNA binding protein, VirE2, coats the DNA sometime during the transfer process, although this interaction may occur after the VirD2-T strand and VirE2 have been independently translocated to the plant cell (8, 13, 34, 42).

The processing of T-DNA and its movement from Agrobacterium to plants is similar to the conjugal transport of a variety of plasmids in gram-negative bacteria (reviewed in references 12, 29, and 48). During conjugal transfer, single-stranded plasmid DNA is thought to move from donor to recipient through a membrane-spanning pore encoded by the transfer (tra) genes of conjugative plasmids. Sequence comparisons have shown that the virB genes of A. tumefaciens, which most likely produce a membrane-localized multimeric protein channel for T-DNA export, are quite similar to several conjugal transport operons (12). The virB genes of A. tumefaciens also have significant sequence homology with the pil genes of Bordetella pertussis (20, 39, 46), the products of which are required for the export of the six-subunit pertussis toxin. Given this similarity to a protein export system, it is perhaps not surprising that in addition to translocating DNA-protein complexes the VirB transport apparatus also appears to mediate the movement of proteins. Strains mutant in VirE2 (34) or VirF (30) can be complemented for tumorigenesis by coinfection with a helper strain that carries an intact vir region but lacks a T region, suggesting that both VirE2 and VirF proteins can be exported from Agrobacterium independently of the VirD2-T strand. Extracellular complementation assays have also shown that virE mutants strains are capable of moving an uncoated VirD2-T strand out of the bacterium into the plant cell (30, 34). To date, all of the VirB proteins tested, with the exception of VirB1, are essential for the movement of T-DNA (5, 17, 43). VirD2-T strands (8), VirE2 (8, 14), and VirF (30) from Agrobacterium to plant cells. These observations suggest that the VirB complex is a multifunctional translocation apparatus that recognizes and exports diverse substrates, most likely based on information contained within their protein component.

The hypothesis that the mechanisms of T-DNA transfer and conjugation are functionally related is further supported by the observation that plasmid RSF1010, a mobilizable, broad-host-range plasmid of the IncQ incompatibility group, can be transferred by A. tumefaciens to plant cells (10) in a process that requires the VirB proteins (44). Interestingly, the VirB and VirD4 proteins of A. tumefaciens can also direct the conjugal transfer of RSF1010 between agrobacteria (4), RSF1010, which lacks the border sequences upon which the VirD2 protein acts, carries genes which encode three proteins (MobA, MobB, and MobC) and carries an origin of transfer (oriT), all compactly organized within a 2.9-kb region of the plasmid. Each of these sequences is required for mobilization during conjugation (9, 19). Nicking of the DNA strand at oriT is carried out in a DNA-protein complex, called the relaxosome, by MobA and MobC (36, 49, 50). MobB increases the proportion of molecules specifically nicked at oriT, thereby increasing the efficiency of relaxosome formation (35). After site-specific nicking of one DNA strand, MobA becomes covalently attached to the 5’ end of the nicked strand and transfer of the single-stranded DNA into the recipient bacteria is initiated (6,
 DH5α  λ− φ80lacZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK− mK−) supE44 thi-1 gyrA relA1  GIBCO BRL  40
 S17-1  Chromosomally integrated mut genes of pRP4; Spf  23
 HB101  F− hsdS20 (rK− mK−) supE44 recA1 ara-14 (39) gillK2 lacY1 proA2 rpsL120 (Smr) y15 metI1 rnh−  Contech  8

**A. tumefaciens**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAD1285</td>
<td>Broad-host-range vector derived from high-copy-number mutant of pTJS75; IncP Cb r Tet’</td>
<td>This study</td>
</tr>
<tr>
<td>pAJ1</td>
<td>mobA1 derivative of pJB31</td>
<td>This study</td>
</tr>
<tr>
<td>pAJ5</td>
<td>2.9-kb Aval fragment containing the mob region from pAJ1 cloned into pBluescript; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pAJ6</td>
<td>oriT derivative of pJB31</td>
<td>This study</td>
</tr>
<tr>
<td>pJK19</td>
<td>pBluescript (Stratagene, La Jolla, Calif.)</td>
<td>Clontech</td>
</tr>
<tr>
<td>pK20</td>
<td>Kan’ Sp’</td>
<td>This study</td>
</tr>
<tr>
<td>pJB31</td>
<td>RSF1010-derived broad-host-range vector; IncQ Sp’</td>
<td>This study</td>
</tr>
<tr>
<td>pKT231</td>
<td>RSF1010 derivative; IncO Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS1</td>
<td>5.7-kb XmnI/PstI fragment containing the mob and rep regions from pKT231 cloned into pBluescript; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS2</td>
<td>5.7-kb XmnI/PstI fragment containing the mob and rep regions from pAJ6 cloned into pBluescript; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS7</td>
<td>2.9-kb Aval fragment containing the wild-type mob region from pAJ6 cloned into pBluescript; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS8</td>
<td>5.7-kb XmnI/PstI fragment containing the mob and rep regions from pAJ6 cloned into pBluescript; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS17</td>
<td>pAD1295 with nos-NPTII plant-selectable marker; IncP Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS18</td>
<td>2.9-kb Aval fragment containing the wild-type mob region from pKT231 cloned into pLS17; Cb’</td>
<td>This study</td>
</tr>
<tr>
<td>pLS50</td>
<td>PstI Gm’ gene fragment from pML122 cloned into pJB20; IncW Tet’ Gm’</td>
<td>This study</td>
</tr>
<tr>
<td>pML122</td>
<td>RSF1010-derived broad-host-range vector; Gm’ Tet’</td>
<td>This study</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Self-transmissible, ColE1 replicon, trn functions of pRK2; IncP Kan’</td>
<td>This study</td>
</tr>
<tr>
<td>pS2132</td>
<td>Broad-host-range plasmid with lac promoter and lacI; IncP Tet’</td>
<td>This study</td>
</tr>
<tr>
<td>pUCD2</td>
<td>Broad-host-range cloning vector; IncW Cb’ Kan’ Sp’ Tet’</td>
<td>This study</td>
</tr>
</tbody>
</table>
These plasmids were then tested for their capacity to mobilize into recipient strain A348 that contained pLS50, a GmR nonmobilizable plasmid, during incubations on minimal medium containing the vir inducer acetoxycesiogen (AS) (Aldrich Chemical). The conjugation experiments were carried out as previously described (3). Bacteria were then washed from the agar and plated on medium containing spectinomycin and gentamicin or medium containing carbenicillin and gentamicin to select for transconjugants. Donors were quantitated by growth on spectinomycin- or carbenicillin-containing medium, and recipients were quantitated by growth on gentamicin-containing medium. Colonies were scored after 3 days.

Virulence assays. Virulence assays using Kalanchoe daigremontiana were carried out as described previously (42) with strain A348 carrying the various RSF1010 derivatives and with A348 and with A348(pBJ31) as positive and negative controls, respectively. All inoculations were scored for the level of tumor formation after 14, 21, and 28 days. Nicotiana tabacum cv. Havana 42S was used for all tobacco leaf square transformation assays as previously described (2). Leaf squares were scored for tumor growth 10 days after cocultivation with the Agrobacterium strains. The ability of various RSF1010 derivative plasmids to block the capacity of disarmed (no T-DNA) strain LBA4404 to serve as a VirE2 donor was assayed by infecting leaf square explants with a 1:1 mixture (each at an optical density at 600 nm [	ext{OD}_{600}] of 0.5) of LBA4404 carrying the various plasmids and virE2 mutant A348::virE2. After 2 days of cocultivation on hormone-free MS medium (31) containing 100 \(\mu\text{M}\) AS, the leaf squares were washed and transferred to selection (hormone-free MS) medium. Colonial scores were taken after 14 days.

RESULTS

The MobA protein and oriT nic site of pBJ31 are necessary for plasmid movement between E. coli strains. Our earlier results showed that the presence of an RSF1010 derivative in A348 blocked this strain's ability to transform Kalanchoe and tobacco (44). We sought to test the hypothesis that the conjugal intermediate of this plasmid was responsible for the observed inhibition. Therefore, mutations predicted to abolish mobilization were constructed by altering either the mobA coding sequence or the nic site of oriT of RSF1010. Previously published data on RSF1010 (10) have shown that oriT and the entire mob region are within a 2.9-kb AvaI fragment (Fig. 1A) and that a mutation at the AccI site in the mobA gene leads to a nonfunctional mob region (Fig. 1B). In addition, Bhattacharjee et al. have shown, using in vitro assays for cleavage of single-stranded oriT sequence by purified MobA* B-galactosidase hybrid protein, that two G-to-A transitions in bases bracketing the nic site at the oriT of plasmid R162 (nearly identical to RSF1010) decrease cleavage by at least 90% (7) (Fig. 1C). To confirm that mob regions with mutations at these sites no longer support conjugation between E. coli strains, the mob region of the RSF1010 derivative pBJ31 was mutagenized (see Materials and Methods for details) so that it contained a frame-shift mutation at the 5′ end of mobA (yielding mobA1 in pAJ1) or the transition mutations at oriT (yielding oriT1 in pJ6). The 2.9-kb AvaI fragments carrying the mutagenized or wild-type mob regions were then cloned into pBluescript (Stratagene), a small nonmobilizable E. coli plasmid, to create pAJ5 (mobA1), pLS7 (oriT1), and pLS9 (wild-type mob region). Whereas pBluescript carrying the wild-type AvaI fragment (pLS9) is mobilizable at a high frequency from E. coli S17-1 (carrying the tra genes of RP4 in the chromosome) to HB101 (1.7 ± 0.2 transconjugants/donor input [5 × 10^9 bacterial]) pAJ5 (mobA1) is very poorly mobilized (4.8 × 10^{-3} ± 2 × 10^{-3} transconjugants/donor input [5 × 10^8 bacteria]) and pLS7 (oriT1) is essentially nonmobilizable (no transconjugants). (A total of 5 × 10^{6} HB101 bacteria were used as the recipient cells for each experiment.) In the case of pAJ5 a very small number of transconjugants were observed, presumably because the function of MobA can be partially replaced by a gene encoded by the host bacterium (10). Similar results were observed when DH5α strains containing the pBluescript derivatives, as well as the helper plasmid pRK2013 that contains the tra genes of RK2 (21), were used as the donor (data not shown).

The MobA protein and oriT nic site of pBJ31 are necessary for plasmid movement between Agrobacterium strains. In addition to being mobilizable between E. coli strains, RSF1010 can be transferred between Agrobacterium strains in a process that is mediated by the Ti-borne VirB and VirD4 proteins (4, 22) rather than the Ti-borne Tra proteins (16). To demonstrate that pBJ31 derivatives with mutations in mobA or oriT are no longer capable of using the virB-encoded transfer system, strains carrying the mutated RSF1010 derivatives, pAJ1 (mobA1) and pAJ6 (oriT1), were tested for VirB-dependent movement between Agrobacterium strains. In these experiments, spectinomycin-resistant pBJ31, pAJ1, and pAJ6 were transformed into the tumorigenic strain A348 and tested for their ability to be mobilized to a gentamicin-resistant recipient. Table 2 shows
that little if any pAJ1 or pAJ6 transfer was observed compared to that of the wild-type pJB31.

A third set of plasmids was constructed to determine which regions of RSF1010 are sufficient to create a plasmid that is mobilizable through the *Agrobacterium* VirB transfer apparatus. The 2.9-kb *Ava*I fragment (Fig. 1A) that conferred to pBluescript the capacity to be mobilized between *E. coli* strains was cloned into an IncP broad-host-range vector, pLS17, which cannot be mobilized between agrobacteria via the VirB proteins. Surprisingly, the resultant fusion plasmid, pLS18, could not be efficiently mobilized between agrobacteria. The transfer frequency was approximately 500 times less than that of the wild-type RSF1010 derivative pJB31 (Table 2). While extremely inefficient, this transfer was reproducible and required AS (data not shown).

To determine the minimum amount of RSF1010 required for high-efficiency transfer between *Agrobacterium* strains, a 5.7-kb *Pst*I/XmnI fragment (Fig. 1A), containing the *mob* region along with the *rep* genes necessary to create a plasmid that could be maintained in agrobacteria, was cloned into pBluescript to create pLS1. The *mob* and *rep* regions of pAJ1(*mobA1*) and pAJ6(*oriT1*) were also cloned into pBluescript to create pLS2 and pLS8, respectively. These hybrid plasmids were then tested for their ability to mobilize between agrobacteria. Only pLS1, which carries the wild-type *mob* region, was capable of conjugal movement (Table 2).

**pJB31 MobA protein and the *nic* site at *oriT* are necessary for inhibition of tumorigenesis.** We next tested the effect of the RSF1010 derivative plasmids pAJ1 (*mobA1*) and pAJ6 (*oriT1*) on the virulence of wild-type strain A348. Assays using *K. daigremontiana* leaves (data not shown) as well as tobacco leaf disk assays (Fig. 2) showed that virulence, as demonstrated by tumor formation, was inhibited by the wild type (pJB31) but not by the nonmobilizable (pAJ1 and pAJ6) RSF1010 derivatives. These results indicate that both the MobA protein and the *oriT nic* site are essential to the inhibition of virulence. Because the nonmobilizable plasmid pAJ6 is mutant for *oriT* but wild type for MobA, these results also rule out the possibility that the MobA protein itself can inhibit virulence independent of the transferred intermediate. Therefore, by separately mutating the MobA coding sequence and the *oriT nic* site of pJB31, we have shown that a functional *mob* region is necessary for the inhibition of virulence and that inhibition of virulence correlates with the capacity of the plasmid to be mobilized between *A. tumefaciens* strains via the VirB pore.

We also determined that tumor formation was not affected by pLS18, a fusion of the *mob* region of RSF1010 with the IncP plasmid pLS17. In contrast, the mobilizable plasmid pLS1, consisting of the wild-type *mob* region plus *rep* genes of RSF1010 in pBluescript, did prevent tumorigenesis (Fig. 2), further suggesting that the capacity to be efficiently mobilized through the VirB transfer apparatus correlates directly with virulence inhibition.

**Both MobA and the *oriT nic* site of pJB31 are necessary for inhibition of VirE2 transfer.** Having demonstrated that a mobilizable version of the RSF1010 derivative pJB31 was necessary to inhibit virulence, we next sought to determine whether the capacity for mobilization was necessary to inhibit the capacity of an *Agrobacterium* strain to serve as a VirE2 donor in the extracellular complementation of virE2 mutants (8, 34). In the assays used in this study, plant tissues are exposed to a mixture of two strains, a virE mutant strain containing the wild-type T-DNA or a standard binary vector, pEND4K, carrying a plant-expressible neomycin phosphotransferase gene (*nosP-nptII*) and a strain producing VirE2 but lacking the T-DNA or the binary vector. The stable transformation of plant cells by the T-DNA from either of the virE2 mutant T strand donors requires coinfection with the helper strain wild type for virE. Using such extracellular complementation assays, we previ-

### TABLE 2. Mobilization of RSF1010 derivatives between *Agrobacterium* strains

<table>
<thead>
<tr>
<th>Donor strain</th>
<th>Feature(s)</th>
<th>Donor output (10^5)</th>
<th>No. of trans-conjugants</th>
<th>Mean no. (±SD) of transconjugants/ output donor</th>
<th>% of A348(pJB31) transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>A348(pJB31)</td>
<td>Wild-type <em>mob</em> region</td>
<td>5.2</td>
<td>210,000.0</td>
<td>4.0 × 10^5 ± 0.4 × 10^5</td>
<td>100.0</td>
</tr>
<tr>
<td>A348(pAJ1)</td>
<td>pJB31 with <em>mobA1</em></td>
<td>10.3</td>
<td>4.7</td>
<td>4.6 × 10^10 ± 6.5 × 10^10</td>
<td>0.01</td>
</tr>
<tr>
<td>A348(pAJ6)</td>
<td>pJB31 with <em>oriT1</em></td>
<td>12.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>A348(pLS1)</td>
<td>IncQ <em>mob-rep</em> region in pBS</td>
<td>6.3</td>
<td>205,000.0</td>
<td>3.3 × 10^5 ± 0.3 × 10^5</td>
<td>82.5</td>
</tr>
<tr>
<td>A348(pLS2)</td>
<td>pLS1 with <em>mobA1</em></td>
<td>8.0</td>
<td>3.3</td>
<td>4.2 × 10^10 ± 5.2 × 10^10</td>
<td>0.001</td>
</tr>
<tr>
<td>A348(pLS8)</td>
<td>pLS1 with <em>oriT1</em></td>
<td>1.4</td>
<td>1.7</td>
<td>1.0 × 10^9 ± 1.8 × 10^9</td>
<td>0.002</td>
</tr>
<tr>
<td>A348(pLS17)</td>
<td>IncP vector</td>
<td>2.1</td>
<td>0.7</td>
<td>3.4 × 10^10 ± 2.9 × 10^10</td>
<td>0.001</td>
</tr>
<tr>
<td>A348(pLS18)</td>
<td>pLS17 and RSF1010 <em>mob</em> region</td>
<td>0.4</td>
<td>31.0</td>
<td>7.3 × 10^8 ± 0.8 × 10^8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Data are the means of triplicate determinations from a single experiment. A total of three independent experiments with similar results were performed. Totals of 2 × 10^6 donors and 10^6 recipients (A348[pLS50]) were used as input in the conjugation mix. The number of recovered (output) recipients after 3 days’ growth at 25°C on induction medium was approximately 4 × 10^6 cells.
the fact that it can, itself, be transferred from these *Agrobacterium* cells to plant cells. Additionally, we found that this plasmid also blocks the capacity of this strain to serve as a VirE2 donor in extracellular complementation assays (8, 44) but barely inhibits the transfer of the VirD2-T strand from virE2 mutants into plant cells. These results suggest that disruption of VirB-mediated VirE2 transfer by RSF1010 is largely responsible for this plasmid’s effects on virulence. Here we show that RSF1010 derivatives with mutations in either the *mob4* coding sequence or the *nic* site at oriT cannot be mobilized between agrobacteria in a virB-dependent manner (Table 2) and no longer inhibit virulence (Fig. 2) or the transfer of VirE2 (Fig. 3) from agrobacteria to plants. We have also shown that a small nonmobilizable *E. coli* plasmid carrying the *rep* genes as well as the *mob* region of RSF1010 (Fig. 1) is efficiently mobilized between *Agrobacterium* strains (Table 2) and inhibits *Agrobacterium* virulence (Fig. 2).

Intriguingly, we were unable to prove that the mobilization region of RSF1010 is sufficient to inhibit tumorigenesis or VirE2 movement when it is carried in an IncP broad-host-range vector. The promoters and coding sequences of *MobA*, *MobB*, and *MobC*, as well as the sequences for oriT, the origin of transfer, and *oriV*, the vegetative origin of replication, were isolated as a 2.9-kb *AvaI* fragment (Fig. 1A) and cloned into pLS17, a high-copy-number IncP plasmid (18) carrying a plant-selectable marker, to create pLS18. In contrast to the RSF1010 derivative pJB31, however, the hybrid plasmid was mobilizable only at a very low frequency between *Agrobacterium* strains (*virB*-dependent conjugation rates were at least 500-fold less than that of pJB31) (Table 2). Similar results were obtained when the 2.9-kb *mob* region was cloned into two other broad-host-range vectors, pSW213 (IncP) and pUCD2 (IncW) (data not shown). It is possible that local conformation of the hybrid plasmid helix affects the activity of the RSF1010 *oriT* or of the adjacent *mob* gene promoters (30a). Because vegetative and transfer modes of replication may be coordinated (26, 32), competition between *oriV* or *oriT* from RSF1010 with those of the IncW and IncP plasmids may prevent efficient relaxosome assembly and formation of conjugal intermediates. None of the three RSF1010-broad-host-range hybrid plasmids tested demonstrated an effect on virulence or VirE2 transfer (Fig. 2 and data not shown), again supporting the hypothesis that the conjugal intermediates are required for inhibition of *virB*-mediated transport.

Plant transformation assays did, however, show that pLS18, but not pLS17, was capable of movement into plant cells (data not shown). These results confirm the findings of Buchanan-Wollaston et al. (10), who showed that the *mob* region of RSF1010 can mediate the transfer of an IncP broad-host-range plasmid from *Agrobacterium* into plant cells. The level of conjugal efficiency exhibited by pLS18 is evidently sufficient to form complexes necessary for transfer to plants but is not sufficient to outcompete VirE2 for available export sites.

How could the RSF1010 transfer intermediate block or disrupt access of VirE2, and to a lesser extent that of the VirD2-T strand, to the VirB pore? Certainly the relative abundance of RSF1010 in agrobacteria (at least 20 copies/cell) may contribute to this. However, we have shown that high copy number of the plasmid alone, without the capacity to mobilize efficiently via the VirB transport apparatus, is not sufficient for inhibition. The mutant high-copy-number RSF1010 derivatives pAJ1 and pAJ6 do not affect A348 virulence. Additionally, the poorly

![FIG. 3. Effects of different RSF1010 derivatives on the capacity of *Agrobacterium* sp. strain LBA4404 to serve as a VirE2 donor. *N. tabacum* cv. Havana 425 leaf explants were infected with a VirE2 donor strain (LBA4404 without or with RSF1010 derivative plasmids) either in the absence (−) or presence (+) of the T-strand donor strain A348::virE2 for 2 days, as described in Materials and Methods, and then transferred to selection medium. The mean numbers of tumors per explant ± standard errors (*n* = 18 leaf pieces) were determined after a 10-day incubation on selection medium.

DISCUSSION

We demonstrated previously that the IncO broad-host-range plasmid RSF1010 inhibits the virulence of strain A348 despite

Intriguingly, we were unable to prove that the mobilization region of RSF1010 is sufficient to inhibit tumorigenesis or VirE2 movement when it is carried in an IncP broad-host-range vector. The promoters and coding sequences of *MobA*, *MobB*, and *MobC*, as well as the sequences for *oriT*, the origin of transfer, and *oriV*, the vegetative origin of replication, were isolated as a 2.9-kb *AvaI* fragment (Fig. 1A) and cloned into pLS17, a high-copy-number IncP plasmid (18) carrying a plant-selectable marker, to create pLS18. In contrast to the RSF1010 derivative pJB31, however, the hybrid plasmid was mobilizable only at a very low frequency between *Agrobacterium* strains (*virB*-dependent conjugation rates were at least 500-fold less than that of pJB31) (Table 2). Similar results were obtained when the 2.9-kb *mob* region was cloned into two other broad-host-range vectors, pSW213 (IncP) and pUCD2 (IncW) (data not shown). It is possible that local conformation of the hybrid plasmid helix affects the activity of the RSF1010 *oriT* or of the adjacent *mob* gene promoters (30a). Because vegetative and transfer modes of replication may be coordinated (26, 32), competition between *oriV* or *oriT* from RSF1010 with those of the IncW and IncP plasmids may prevent efficient relaxosome assembly and formation of conjugal intermediates. None of the three RSF1010-broad-host-range hybrid plasmids tested demonstrated an effect on virulence or VirE2 transfer (Fig. 2 and data not shown), again supporting the hypothesis that the conjugal intermediates are required for inhibition of *virB*-mediated transport.

Plant transformation assays did, however, show that pLS18, but not pLS17, was capable of movement into plant cells (data not shown). These results confirm the findings of Buchanan-Wollaston et al. (10), who showed that the *mob* region of RSF1010 can mediate the transfer of an IncP broad-host-range plasmid from *Agrobacterium* into plant cells. The level of conjugal efficiency exhibited by pLS18 is evidently sufficient to form complexes necessary for transfer to plants but is not sufficient to outcompete VirE2 for available export sites.

How could the RSF1010 transfer intermediate block or disrupt access of VirE2, and to a lesser extent that of the VirD2-T strand, to the VirB pore? Certainly the relative abundance of RSF1010 in agrobacteria (at least 20 copies/cell) may contribute to this. However, we have shown that high copy number of the plasmid alone, without the capacity to mobilize efficiently via the VirB transport apparatus, is not sufficient for inhibition. The mutant high-copy-number RSF1010 derivatives pAJ1 and pAJ6 do not affect A348 virulence. Additionally, the poorly
mobilizable hybrid plasmid pLS18, which also had no effect on A348 virulence, is most likely present in agrobacteria at a very high copy number, around 30 to 45 copies per cell (18). An alternative is that the transferred intermediate of RSF1010 has advantages over VirE2 (or the VirD2-T strand) in interacting with the VirB pore. Mobilizable plasmids such as RSF1010 can be isolated from donor cells as relaxosomes, DNA-protein complexes that exist in an equilibrium of cleaved and uncleaved nic sites at the oriT. The formation of the relaxosome complex appears to be constitutive and does not depend on the presence of a recipient cell or any other conjugative trigger (reviewed in reference 28). Free, single-stranded DNA transfer intermediates of mobilizable broad-host-range plasmids have not been found in donor cells, suggesting that plasmid DNA movement is coupled to processing of the transferred intermediate (28). In contrast, vir-inducing conditions are required for the production of Vir proteins and the formation of single-stranded T-DNA transfer intermediates that have been detected in the donor A. tumefaciens cell (47). Thus, the relaxosome may be more readily available as potentially limiting DNA movement is coupled to processing of the transferred intermediate. The results of this and previous studies suggest that inefficient mobilization of T-DNA by site-directed mutagenesis. Cell 27:143–153.


