The Use of pNJ5000 as an Intermediate Vector for the Genetic Manipulation of *Agrobacterium* Ti-plasmids

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The use of broad-host-range plasmids derived from RP4 as intermediate vectors for the transfer of narrow-host-range recombinant plasmids from *Escherichia coli* to *Agrobacterium tumefaciens* as a preliminary to marker exchange is described. Recombinant plasmids having a ColE1 type origin were linked to the RP4 derivative. Cointegrate formation appeared to take place by RecA-independent, homologous recombination within a short piece of DNA derived from the β -lactamase gene of Tn1/Tn3 carried by both vector components, so that it never disrupted the recombinant portion of the construction. pNJ5000 provides an unstable intermediate vector for use in marker exchange experiments, while its stable relative pNJ1020 provides a carrier for use in binary vector systems.

INTRODUCTION

Several descriptions exist of intermediate vectors based on broad-host-range IncP plasmids which can be used to carry modified Ti-plasmid segments, cloned and maintained in *Escherichia coli* in ColE1 derivatives, into *Agrobacterium tumefaciens*, where the modification can be transferred to the resident Ti-plasmid by a reciprocal double crossover resulting in a replacement of the wild-type region with the modified region ('homogenotization' or 'marker exchange') (Garfinkel *et al.*, 1981; Matzke & Chilton, 1981; Ruvkun & Ausubel, 1981). The IncP plasmid supplies mobilization functions (*mob*) and an origin of replication (the *bom* site) since, although ColE1 Mob⁻ Bom⁺ derivatives (e.g. pBR322: Sutcliffe, 1978; Peden, 1983) can be transferred directly to *A. tumefaciens* by using trans-mobilization (Warren *et al.*, 1978; Comai *et al.*, 1983; Van Haute *et al.*, 1983), they will not replicate in the alternative host. However, trans-mobilization cannot be used with the safer, GMAG/NIH approved vectors pAT153 (Twigg & Sherratt, 1980), pBR328 and pBR327 (Soberon *et al.*, 1980), all of which have the Mob⁻ Bom⁻ phenotype. These vectors can be introduced into *A. tumefaciens* by transformation using the freeze-thaw technique of Holsters *et al.* (1978), but this procedure is inefficient. A conjugative intermediate system has been described which uses a non-conjugative IncW plasmid which, like the Mob⁻ Bom⁺ ColE1 derivatives, can be mobilized into *A. tumefaciens* using helper functions supplied by the donor strain, but which has the advantage of being stable in *Agrobacterium* (Leemans *et al.*, 1981).

Possibly the most serious drawback of using recombinants based on ColE1 is that, given lack of maintenance of such replicons in *Agrobacterium*, the most frequent event leading to marker recovery is a single crossover which inserts the whole recombinant into the Ti-plasmid, rather than the reciprocal double crossover resulting in incorporation of the modified region (Comai *et al.*, 1983). While the single crossover event is acceptable for gene insertion (Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983), it is of no use for generating deletions of specific regions. Under such circumstances, selecting for the second crossover can be difficult, depending on the selectable markers available.

Table 1. *Bacterial strains*

Species	Strain	Phenotype/genotype	Reference
<i>Escherichia coli</i>	ED8767	<i>supE supF hsdS Met⁻ recA56</i>	Murray <i>et al.</i> (1977)
	W3110	Thy ⁻	Campbell <i>et al.</i> (1978)
	HB101	Pro ⁻ Gal ⁻ <i>rpsL recA1</i>	Boyer & Roulland-Dussoix (1969)
	UNF510	:: Tn5 <i>rpsL His⁻ Trp⁻ Lys⁻ Lac⁻</i>	Merrick <i>et al.</i> (1978)
<i>Agrobacterium tumefaciens</i>	C58nal	<i>nal-100*</i>	This paper
	T37spec	<i>spc-100*</i>	This paper
	HRR1	<i>rif-100*</i>	This paper

* Concentration level ($\mu\text{g ml}^{-1}$) for selection.

Table 2. *Plasmids and transposons*

Plasmid	Antibiotic-resistance phenotype	Resistance ($\mu\text{g ml}^{-1}$)		Reference
		<i>E. coli</i>	<i>A. tumefaciens</i>	
pBR322	Cb	200	200	Sutcliffe (1978)
	Tc	20	5*	
pAT153	Cb	200	200	Twigg & Sherratt (1980)
	Tc	20	5*	
pPH1J1	Ge	10	10	Hirsch & Beringer (1984)
	Sp	50	50	
	Cm	20	20	
pKan1	Cb	200	200	Pearson (1984)
	Km	20	20	
pSZ707	Cb	200	200	This paper
	Km	20	20	
pTiT37	None			Sciaky <i>et al.</i> (1978)
pTiC58	None			Sciaky <i>et al.</i> (1978)
Transposon				
Tn5	Km	20	20	Rothstein <i>et al.</i> (1980)
Tn903	Km	20	20	Grindley & Joyce (1980)

* Resistances which only operate in strain T37.

Where conjugative, stable intermediate vectors have been used, fusion with the T-region recombinant plasmid has been achieved by *in vitro* recombination, a system which offers only limited flexibility, defined by the restriction maps of the respective components. We therefore sought an alternative system for the formation of an intermediate vector which would still offer a simple selection for marker exchange in *Agrobacterium*. The core of this system is provided by pNJ5000, a derivative of RP4 which is unstable in RecA⁺ hosts (Grinter, 1983).

METHODS

Bacterial strains and plasmids. These are listed in Tables 1 and 2. *E. coli* strains were grown at 37 °C in L-broth containing Difco Bactotryptone (10 g l⁻¹), Difco yeast extract (5 g l⁻¹), NaCl (5 g l⁻¹), D-glucose (1 g l⁻¹), or on nutrient agar (Oxoid) if kanamycin selection was to be applied. *Agrobacterium* strains were grown at 25 °C in complete medium containing Difco tryptone, Difco yeast extract (5 g l⁻¹), (3 g l⁻¹).

Mating conditions. Conjugations were done by mixing overnight cultures of bacterial strains in a ratio of 1:1, donor:recipient (1:2 for *E. coli* × *Agrobacterium* crosses), and air drying 0.5 ml samples on cellulose nitrate filters (2.3 cm diameter). Where at least one of the parents was *A. tumefaciens*, the filters were incubated on complete agar plates at 25 °C for 1–3 d. When both parents were *E. coli*, the filters were incubated on L-agar at 37 °C for 15–20 h. After incubation, the bacteria was washed off the filters in water and plated on selective medium at appropriate dilutions. *Agrobacterium* recipient strains were tested for keto-lactose metabolism according to Bernaerts & DeLey (1963).

Selection of tetracycline sensitive cells. Direct selection of tetracycline-sensitive, kanamycin-resistant *Agrobacterium* cells (cells which had lost the vector but retained the marker for homogenote formation) was done by a modification of the techniques of Bochner *et al.* (1980) and Maloy & Nunn (1981). The medium of Maloy & Nunn (1981) was made containing chlortetracycline (20 mg l^{-1} ; Sigma), which was then inactivated by autoclaving at 121°C for 1 h, fusaric acid ($12 \text{ } \mu\text{g ml}^{-1}$) and zinc chloride (0.1 M). This medium greatly enriched for tetracycline-sensitive cells. The addition of kanamycin sulphate ($50 \text{ } \mu\text{g ml}^{-1}$) selected further for these cells which retained the marker for homogenote formation.

Plasmid profiles of Agrobacterium strains. These were examined by analysing small-scale plasmid preparations, made according to Kerr & Roberts (1972) or Machida & Ikeda (1983), on 0.7% (w/v) agarose gels as described by Meyers *et al.* (1976). Gels prepared in this manner were treated with 2% (v/v) HCl for 15 min and transferred to Genescreen (New England Nuclear) as described by Hepburn *et al.* (1983a).

Total DNA preparations from A. tumefaciens. Cells from a 48 h culture (50 ml) were harvested by centrifugation and resuspended in 12 ml 50 mM-Tris/HCl (pH 8.0), 20 mM-EDTA (= TE8 buffer) to which were added SDS (0.01%, w/v, final concentration) and predigested Pronase B ($400 \text{ } \mu\text{g ml}^{-1}$ final concentration; Calbiochem). The resuspended cells were then lysed at 37°C for 1 h. The volume was increased to 50 ml with TE8 buffer and the lysate was extracted once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), and once with an equal volume of phenol saturated with 10 mM-Tris/HCl (pH 8.0) and containing 0.1% (w/w) 8-hydroxyquinoline. Total nucleic acids were precipitated from the final aqueous phase by treatment with 2 vols ethanol at -20°C for 0.5 h. The crude DNA preparation was purified on CsCl gradients and stored frozen in 10 mM-Tris/HCl (pH 8.0), 1 mM-EDTA.

Recombinant DNA and molecular techniques. The construction of the recombinant plasmids and their analysis by restriction digestion or hybridization were as described by Hepburn *et al.* (1983a, b).

RESULTS

Construction of T-region recombinant plasmids

The starting points for the marker exchange system are recombinants comprising modified segments of a Ti-plasmid cloned in pBR322 or its Bom^- derivatives pAT153 (Twigg & Sherratt, 1980) or pBR327 (Soberon *et al.*, 1980). Since our long term interests lie in the use of the Ti-plasmid as a vehicle for modifying higher plants, we have concentrated on Ti-plasmid segments derived from within and around the T-region. Two such clones will be used to show aspects of the system. Other minor variations will be described later.

Tn5 (Rothstein *et al.*, 1980) insertions into the recombinant plasmid pSZ643 (Hepburn *et al.*, 1983a), which contains *Hind*III fragments 23 and 31 of pTiC58 (Depicker *et al.*, 1980), were obtained using *E. coli* strain UNF510 (Merrick *et al.*, 1978) as the Tn5 donor and a single step selection method to isolate the mutant. Cells of UNF510 containing the recombinant plasmid were spread as a lawn on nutrient agar containing kanamycin ($15 \text{ } \mu\text{g ml}^{-1}$), and a drop of kanamycin (5 mg ml^{-1}) was applied to the centre of the plate. Isolated colonies within the cleared zone in the middle of the plate result from Tn5 insertions into the recombinant plasmid which, as it is present in many copies, confers high level resistance to kanamycin on its hosts. The recombinant plasmid, pSZ643::Tn5-19, carries the transposon approximately 470 bases to the right of the T-region right border (Hepburn *et al.*, 1983a). The *Bgl*II/*Bam*HI fragment from Tn5 (1.5 kb of DNA) which contains the neomycin phosphotransferase II (NPTII) gene but not its promoter (Beck *et al.*, 1982) was isolated from agarose gels and cloned into the *Bam*HI site near the middle of the nopaline synthase gene to give pKan1 (Fig. 1) (Pearson, 1984).

pSZ707 (Fig. 1) was obtained by ligating the right 1.5 kb of DNA from *Hind*III fragment 7, which lies to the left of the T-DNA left border, to *Hind*III fragment 33, which lies to the right of the right T-DNA border (Depicker *et al.*, 1980), using pAT153 as the *E. coli* vector. To provide a marker for the eventual homogenotization event, the central *Pvu*II fragment (1.7 kb) of Tn903, which carries the neomycin phosphotransferase I (NPTI) gene encoding kanamycin resistance (Grindley & Joyce, 1980), was inserted into the *Hind*III site at the junction of the two Ti-plasmid fragments. Since this cloning eliminates the transposition genes and most of the terminal inverted repeat sequences from Tn903, this fragment constitutes an immobile marker at the site of the deletion.

Both the recombinant plasmids contain sequences from the nopaline Ti-plasmid pTiC58. pSZ707 contains NPTI for selection for marker exchange at the site of a large deletion which

Fig. 1. Restriction endonuclease maps of pSZ707 and pKan1. The major components of the plasmids are indicated. *Hind*III fragment numbers are taken from Depicker *et al.* (1980). NPTII is the structural gene for neomycin phosphotransferase II isolated as a *Bgl*II/*Bam*HI fragment from Tn5. NPTI is the central *Pvu*II fragment from Tn903 which includes the entire neomycin phosphotransferase I gene. Arrows indicate the positions of the nopaline synthase gene (*nos*) promoter and the right border of the T-DNA. H, *Hind*III; B, *Bam*HI; S, *Sal*I; H', a fused *Hind*III and *Pvu*II site; B', a fused *Bam*HI and *Bgl*II site.

comprises 14% of its Ti-plasmid parent, while pKan1 contains a functional transposon as a selectable marker approximately 1.8 kb away from a small insertion. One further feature of this latter construction is that the right border of the T-region separates the marker and the DNA insertion.

The intermediate vector and cointegrate formation

To construct pNJ5000, Grinter (1983) used pRP1, a Tn7 insertion into RP4 (Barth & Grinter, 1977), as the starting plasmid. *Pst*I digestion of pRP1 and subsequent ligation of the resultant fragments generated pNJ5000, which contains the two major *Pst*I fragments joined in the normal orientation. pNJ5000 is unstable in a *RecA*⁺ background but still resistant to tetracycline and conjugative to a wide range of Gram-negative bacterial species (Grinter, 1983).

pNJ5000 was conjugally transferred, with an observed frequency of 1%, to *E. coli* strain ED8767 carrying either pKan1 or pSZ707. Cointegrates were selected from the population of ED8767, carrying both a recombinant plasmid and pNJ5000, by conjugation into HB101, and were identified using streptomycin and either carbenicillin or kanamycin. Since the *ColE1*-based recombinant plasmids are not self-mobile, transfer of the resistances encoded by these recombinant plasmids indicates mobilization through cointegrate formation. The frequency of transfer of the recombinant markers when related to the frequency of transfer of the tetracycline resistance marker of pNJ5000 indicates a frequency of cointegrate formation of from 10^{-3} to 10^{-5} , and was generally reproducible for a given recombinant plasmid, suggesting that the DNA sequence carried by the recombinant may affect the process.

In all cases, cointegrate formation had taken place between pNJ5000 and the vector component of the recombinant plasmid. Multiple restriction enzyme digestion of cointegrate plasmids isolated by the STET method of Holmes & Quigley (1981) immediately after cointegrate selection showed the same fragments to be absent in each case, together with the concomitant appearance of an identical fusion fragment. Fig. 2 shows the structure of the cointegrate formed between pKan1 and pNJ5000. The fragments of the two component plasmids missing from the cointegrate are both vector fragments. Using several combinations of restriction enzymes, the regions within which recombination had occurred were localized as lying between 0 kb (*Eco*RI) and 3.9 kb (*Pst*I) on the pNJ5000 map and between 2066 bp (*Pvu*II) and 3601 bp (*Pst*I) on the pBR322 map. Although dissociation occurs at a significant rate,

Fig. 2. Regions of cointegrate formation between pKan1 and pNJ5000. Plasmid DNA isolated by the STET method was digested by restriction enzymes and fractionated on a 1% (w/v) agarose gel in 40 mM-Tris, 20 mM-sodium acetate, 1 mM-EDTA at pH 8.3. The gel tracks show multiple restriction endonuclease digestion profiles of pNJ5000 (lane A), pKan1 (lane C) and a cointegrate between the two (lane B) obtained using the enzymes *Sac*II, *Kpn*I, *Bam*HI and *Eco*RI. The DNA fragments present in the parent plasmids but not in the cointegrate (band 1, lane A and band 2, lane C) are replaced by two new fragments in the cointegrate (bands 3 and 4, lane B) which represent the digestion products of the DNA around the site of cointegrate formation.

resulting in cells containing both the recombinant plasmid and pNJ5000, the persistence of at least some cointegrate could always be demonstrated by conjugation into a rifampicin-resistant derivative of *E. coli* ED8767, using suitable antibiotic selection. Again, transfer of carbenicillin resistance demonstrated the presence of the cointegrate.

Transfer to Agrobacterium and marker exchange

The cointegrate can be mobilized from *E. coli* into *A. tumefaciens* at a frequency of several percent. After transfer, single colonies showing the appropriate resistance phenotypes were analysed to determine their plasmid content and confirm that they contained both the wild-type Ti-plasmid and the cointegrate (Fig. 3, lane F).

The final recombination event between the Ti-plasmid and the cointegrate can be selected in either of two ways depending on the *Agrobacterium* host strain used. The product of the double reciprocal crossover which replaces wild-type Ti-plasmid sequences by modified sequences will result in the insertion of the homogenate marker (NPTI or NPTII in the examples cited) into the Ti-plasmid. It can then be inherited independently of the markers on the ColE1 and pNJ5000 vector components. Since pNJ5000 is unstable in a RecA⁺ background, with a rate of loss in the absence of selection of 5% per generation (Pearson, 1984) after 7 d of continuous exponential culture, no more than 10% of the *Agrobacterium* cells in a liquid culture still carried the cointegrate.

When the host strain is C58, tetracycline sensitive cells can be selected directly in the presence of selection for the kanamycin resistance as described. At least 98% of colonies selected by this method were tetracycline sensitive. Because selection for tetracycline resistance cannot be used with strain T37, an alternative screening procedure was used. After 7 d culture in the absence of selection, the cells were conjugated with an *E. coli* strain carrying pPH1JI (Hirsch & Beringer,

Fig. 3. Plasmids contained by potential modified clones. (a) 0.7% (w/v) agarose gel analyses of small scale plasmid preparations from *A. tumefaciens* strains. Lane F of both panels shows the profile of C58na1 containing the cointegrate between pNJ5000 and pKan1 (Cm, monomer; Cd, dimer), pTiC58 (Ti) and the cryptic plasmid pATC58 (At). Lanes A to E show the plasmid contents of possible homogenote clones selected on the basis of their sensitivity to tetracycline. (b) Bands of hybridization obtained when Southern blots of the two gels were probed with a mixture of pNJ5000 and pBR322 (left panel) and pBR322::Tn5 (right panel). The signal in lane B identifies the only true potential homogenote clone where Tn5 sequences have been retained by the Ti-plasmid but all vector sequences have been lost.

1984). Since pPH1J1 encodes gentamicin resistance and is incompatible with pNJ5000, selection for gentamicin resistance, in addition to selection for the *Agrobacterium* host and the homogenate marker (kanamycin resistance), yields cells where the modified region of the T-DNA is maintained in the absence of the cointegrate, presumably following exchange into the resident Ti-Plasmid. The level of spectinomycin resistance in the T37 host is fivefold higher than the level of spectinomycin resistance encoded by pPH1J1, and hence can be used in the cointegrate elimination process. This method can also be used with *A. tumefaciens* strain C58 either instead of or in addition to the selection for tetracycline sensitivity.

Plasmid analyses on colonies showing the correct resistance phenotype confirmed that they did not contain any cointegrate and that no portions of the vector were present in the Ti-plasmid. The example in Fig. 3 shows the plasmids of derivatives of a C58 strain which originally carried a pKAN1-pNJ5000 cointegrate after selection for loss of tetracycline resistance. Although none of the samples contain either pBR322 or pNJ5000 sequences (Fig. 3, lanes A-E), only one shows the presence of Tn5 sequences in pTiC58 (Fig. 3, lane B). The presence on the plasmid of the kanamycin resistance used to select reciprocal exchange was shown by demonstrating linked conjugal transfer of the marker and the Ti-plasmid to a strain of *Agrobacterium* lacking the Ti-plasmid (strain HRR1).

As a final confirmation of marker exchange, total DNA isolated from C58 carrying a possible homogenate was digested with *Hind*III and *Eco*RI, fractionated on agarose gels, blotted onto Genescreen and probed with pSZ707. Fig. 4 shows that the Ti-plasmid pTiC58-Z707 has indeed lost the whole of the T-DNA. The frequency of marker exchange was of the order of 10^{-6} .

Fig. 4. Predicted map of the modified Ti-plasmid in the region of the deletion. Total DNA from a C58nal strain carrying the plasmid (pTiC58-Z707) was digested with *EcoRI* or *HindIII* and the fractionated products hybridized with pSZ707. The major bands predicted from the map (fragments A, B and C) can be seen. The two small *EcoRI* fragments are not visible on the fractionation shown. The bands marked X show hybridization between the NPTI DNA in the probe and specific fragments of pPH1J1 which is also present in the strain. E, *EcoRI*; H, *HindIII*; S, *SalI*; H', a fused *HindIII* and *PvuII* site.

DISCUSSION

The results presented demonstrate the major features of an intermediate vector based on cointegrate-formation for the modification of *Agrobacterium* Ti-plasmids. To date, we have only used the system to modify the noplaine-type Ti-plasmids pTiC58 and pTiT37, but given recipient *Agrobacterium* strains with suitable antibiotic resistance the system should also be applicable to the octopine-type Ti-plasmids. The two examples given demonstrate the use of pNJ5000 in marker exchange experiments. However, we have used pNJ1020, a plasmid related to pNJ5000, as a vector for the transfer of ColE1-based recombinant plasmids, which constitute one part of a binary system for plant transformation (de Framond *et al.*, 1983; Hoekema *et al.*, 1983). The other part of the binary system is a helper plasmid which has a Ti-plasmid origin of replication and provides the *trans*-acting *vir* functions necessary for transformation but has lost all the T-DNA, including the border repeats (pTiC58-Z707). Cointegrates of pNJ1020 and a recombinant plasmid have been selected as described for pNJ5000 and transferred to *Agrobacterium* containing a helper plasmid. The recombinant plasmids are required to be stably maintained in *Agrobacterium* and selection for carbenicillin can be used to counterselect cells in which dissociation has occurred. pNJ1020 contains one more *PstI* fragment from RP4 than pNJ5000, and this fragment spans map positions 28.3–34.7 kb on the RP4/RP1/RK2 map (Shingler & Thomas, 1984; N. J. Grinter, personal communication). Why this *PstI* fragment should confer stability on pNJ1020 is not clear, since it does not encode any of the known maintenance functions (Thomas, 1983; Shingler & Thomas, 1984).

The feature which makes the system particularly easy to handle is that the cointegrate formation always takes place between pNJ5000 and the vector component of the recombinant plasmid. The localization of this event to the specified regions of pNJ5000 and pBR322 suggests the mechanism for this phenomenon. pBR322 contains 1216 bases derived from the right end of Tn3 which includes one copy of the terminal inverted repeat together with the whole of the β -lactamase gene (Sutcliffe, 1978). As a result of its construction, pNJ5000 contains part of Tn1 (Hedges & Jacob, 1974) which is present on RP4. As Tn1 and Tn3 are similar (Calos & Miller, 1980), the two plasmids are likely to have sequences in common. Given the extent of homology between pNJ5000 and pBR322 we would expect a frequency of recombination between them of 1–5% in RecA⁺ cells (Peterson *et al.*, 1982). As RecA-independent recombination events have been shown to occur at 0.1–1.0% of the frequency of RecA⁺ homologous recombination (Doherty *et al.*, 1983), a RecA-independent recombination frequency between the two plasmids of 10^{-4} – 10^{-6} would be expected. This agrees fairly well with the observed range of frequency of cointegrate formation of 10^{-3} – 10^{-5} .

In the case of the homogenate obtained using pKan1 (pTiC58-Kan1), the marker for homogenate formation (Tn5) is located outside the T-region and, as such, is not transferred to infected plant cells (Pearson, 1984), demonstrating that the foreign gene and the marker for homogenate formation need not be genetically linked through all the stages of the modification and transformation process.

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