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Construction of a Tra⁻ deletion mutant of pAgK84 to safeguard the biological control of crown gall

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Summary. *Agrobacterium radiobacter* strain K84 is used commercially for the biological control of crown gall. It contains the conjugative plasmid pAgK84, which encodes the synthesis of agrocin 84, an antibiotic that inhibits many pathogenic agrobacteria. A breakdown of control is threatened by the transfer of pAgK84 to pathogens, which then become resistant to agrocin 84. A mutant of pAgK84 with a 5.9-kb deletion overlapping the transfer (Tra) region was constructed using recombinant DNA techniques. The *Bam*HI fragment B1 which covers most of the Tra region was cloned in pBR325 and its internal *Eco*RI fragments D1 and H, which overlap the Tra region, were removed, leaving 3.7 kb and 0.5 kb of pAgK84 on either side of the deletion. The latter was increased to 3.3 kb by adding *Eco*RI fragment D2 from a *Bam*HI fragment C clone. The modified pBR325 clone was mobilized into *Agrobacterium* strain NT1 harbouring pAgK84 with a Tn*5* insertion just outside the Tra region but covered by the deletion. A Tra⁺ cointegrate was formed between the Tn*5*-insertion derivative and the pBR325-based deletion construct by homologous recombination. The cointegrate was transferred by conjugation to a derivative of strain K84 lacking pAgK84, in which a second recombination event generated a stable deletion-mutant by deletion-marker exchange. The resultant new strain of *A. radiobacter*, designated K1026, shows normal agrocin 84 production. Mating experiments show that the mutant plasmid, designated pAgK1026, is incapable of conjugal transfer at a detectable frequency.

Key words: *Agrobacterium* – Crown gall – Biological control – Agrocin 84 – Agrocin plasmid transfer

Introduction

Crown gall, a disease affecting many dicotyledonous plants, is caused by strains of *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942 which carry a tumour-inducing (Ti) plasmid (Van Larebeke et al. 1975; Watson et al. 1975). The Ti plasmid transfers a portion of its DNA (T-DNA) into plant cells (Chilton et al. 1977) where it is integrated into plant DNA (Chilton et al. 1980; Willmitzer et al. 1980). The integrated T-DNA directs the synthesis of auxin (Inzé et al. 1984; Schröder et al. 1984) and cytokinin (Akiyoshi et al. 1984), which cause tumorous plant cell proliferation, and the synthesis of opines, novel compounds catabolized by the inciting bacteria (Guyon et al. 1980). Opine catabolism is encoded by regions of the Ti plasmid outside the T-DNA (Holsters et al. 1980; De Greve et al. 1981).

Strains of *A. tumefaciens* whose Ti plasmids direct the synthesis and catabolism of the opines nopaline and agrocinopine are important pathogens of stone fruits, nut trees, roses and other ornamentals. Their ability to catabolize agrocinopine has enabled biological control to be effected for these strains by inoculation of seeds and seedling roots with *A. radiobacter* (Beijerinck and van Delden 1902) Conn 1942 strain K84 (see Kerr and Tate 1984). This non-pathogen colonizes roots and produces an antibiotic, agrocin 84, responsible in major part for the control (Kerr and Htay 1974; Kerr and Tate 1984). Agrocin 84 gains entry to the pathogen via agrocinopine permease (Ellis and Murphy 1981; G.T. Hayman and S.K. Farrand, unpublished results) and is thought to act as a terminator of DNA replication (Murphy and Roberts 1979).

Synthesis of agrocin 84 is encoded by pAgK84, a 47-kb conjugative plasmid (Farrand et al. 1985), which also confers immunity to agrocin 84 (Ellis et al. 1979; Ryder et al. 1987). A breakdown of control by strain K84 is threatened by transfer of pAgK84 to pathogens, which then become resistant to agrocin 84 (Ellis and Kerr 1979; Panagopoulos et al. 1979). This breakdown could be avoided if pAgK84 were made transfer deficient (Tra⁻).

Restriction maps of pAgK84 have been produced (Slota and Farrand 1982; Farrand et al. 1985; Ryder et al. 1987) and the Tra region delimited by transposon mutagenesis (Farrand et al. 1985). Derivatives of strain K84 containing transfer-deficient Tn*5*-insertion mutants of pAgK84 control crown gall as effectively as strain K84, demonstrating the feasibility of using Tra⁻ derivatives (Shim et al. 1987). However, these mutants are not desirable replacements for pAgK84 since they may revert to Tra⁺ by loss of Tn*5* and because Tn*5* carries three different antibiotic resistance genes (Genilloud et al. 1984) which might be transferred to other plant, animal or human pathogenic bacteria. Using recombinant DNA techniques we have constructed a stable Tra⁻ deletion mutant of pAgK84 which has neither of these undesirable properties.

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The strategy for construction of this mutant, based on a technique described by Comai et al. (1983), was as follows: (1) clone a portion of pAgK84 containing the Tra region into a cloning vector unable to replicate in *Agrobacterium*; (2) make a deletion in vitro within the cloned Tra region; (3) introduce the modified clone into *Agrobacterium* where it can be rescued by forming a cointegrate by homologous recombination with pAgK84 that has been marked with Tn*5* in the region covered by the deletion; (4) introduce the deletion into pAgK84 by allowing a second recombination event resulting in deletion-marker exchange, with concomitant loss of the vector and the Tn*5* marker. Basing the application of this strategy on the *Bam*HI (Slota and Farrand 1982) and *Eco*RI (Ryder et al. 1987) maps of pAgK84 (Fig. 1), we decided to clone *Bam*HI fragment B1 of pAgK84 and delete, as a minimum, its internal *Eco*RI fragment D1 to create an asymmetric deletion that overlaps the Tra region but which leaves the adjacent clockwise region encoding agrocin synthesis and immunity (Fig. 1) intact.

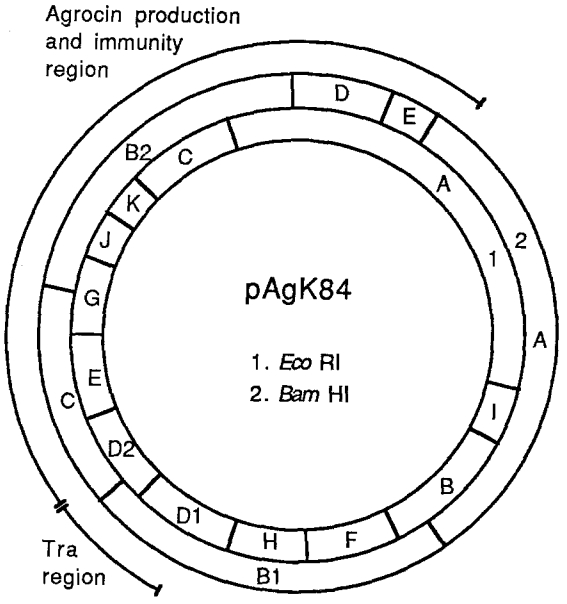

Fig. 1. *Bam*HI and *Eco*RI restriction map of pAgK84 showing the transfer, agrocin synthesis and agrocin immunity regions

Materials and methods

Bacterial strains and plasmids. The bacterial strains and plasmids used are shown in Table 1 and the construction of those developed during the course of this work is described in Results.

Bacterial culture. Escherichia coli was grown at 37° C in either Luria broth (LB, Miller 1972) or yeast extract broth (YEB, Vervliet et al. 1975), as indicated in the text. *Agrobacterium* was grown at 28° C in either YEB, yeast mannitol agar (YMA, Ellis et al. 1979), nutrient agar (NA, Difco or CM1 Oxoid), Bergersen's medium (Bergersen 1961) with 0.2% sodium tartrate, Petit's medium (Petit et al. 1978) with 0.1% $(NH_4)_2 SO_4$ and 0.2% nopaline, or Stonier's medium (Stonier 1960), as indicated in the text. When required, media were solidified with 1.5% Bacto agar, except Bergersen's

Table 1. Bacterial strains and plasmids

Escherichia coli

Strain	Plasmid	Description
HB101	–	*recA*$^-$ cloning recipient (Boyer and Roulland-Dussoix 1969)
–	pBR325	Cloning vector (Bolivar 1978), Ap^r(Cb^r)Cm^r Tc^r
RK2013	pRK2013	HB101 transformed with mobilizing plasmid pRK2013 (Ditta et al. 1980), Km^r
K840	pBR325::*Bam*HI B1	HB101 transformed with pBR325::*Bam*HI B1, Ap^r(Cb^r)Cm^r, this report
K1007	pMHR100	HB101 transformed with pMHR100, Ap^r(Cb^r)Cm^r, this report
K1008	pBR325::*Bam*HI C	HB101 transformed with pBR325::*Bam*HI C, Ap^r(Cb^r)Cm^r, this report
K1022	pDAJ101	HB101 transformed with pDAJ101, Ap^r(Cb^r), this report
K1023	pDAJ102	HB101 transformed with pDAJ102, Ap^r(Cb^r), this report

Agrobacterium

Strain	Biovar	Plasmids[a]	Description
K84	2	pAgK84[b]	Biological control strain producing agrocin 84
K198	1	pTiK27	Indicator strain for agrocin 84 production (Roberts and Kerr 1974)
K434	2	–[b]	Spontaneous mutant of K84 lacking pAgK84 (Shim et al. 1987)
C58-C1RS	1	–	pTi cured C58 (Ellis et al. 1979), Rif^r Str^r
K518	1	–[b]	Transconjugant from K434 × C58-C1RS (A. Kerr unpublished), Rif^r Str^r
NT1	1	–	pTi cured C58 (Watson et al. 1975)
A28	1	pAgK84::Tn*5*A28	NT1 transformed with pAgK84::Tn*5*A28 (Farrand et al. 1985), Km^r Sm^r
K1024	1	pAgK84::Tn*5*A28::pDAJ102	Transconjugant from K1023 × RK2013 × A28, Cb^r Km^r Sm^r, this report
K1025	2	pAgK84::Tn*5*A28::pDAJ102[b]	Transconjugant from K1024 × K434, Cb^r Km^r Sm^r, this report
K1026	2	pAgK1026[b]	Spontaneous derivative of K1025, this report
K1027	2	pAgK1026::pBR325::*Bam*HI C[b]	Transconjugant from K1008 × RK2013 × K1026, Cb^r Cm^r, this report
K1028	2	pAgK84::pBR325::*Bam*HI C[b]	Transconjugant from K1008 × RK2013 × K84, Cb^r Cm^r, this report

[a] All strains of *Agrobacterium* listed contain a large cryptic plasmid in addition to the plasmids indicated
[b] These strains of *Agrobacterium* also harbour pAtK84b which confers the ability to catabolize nopaline

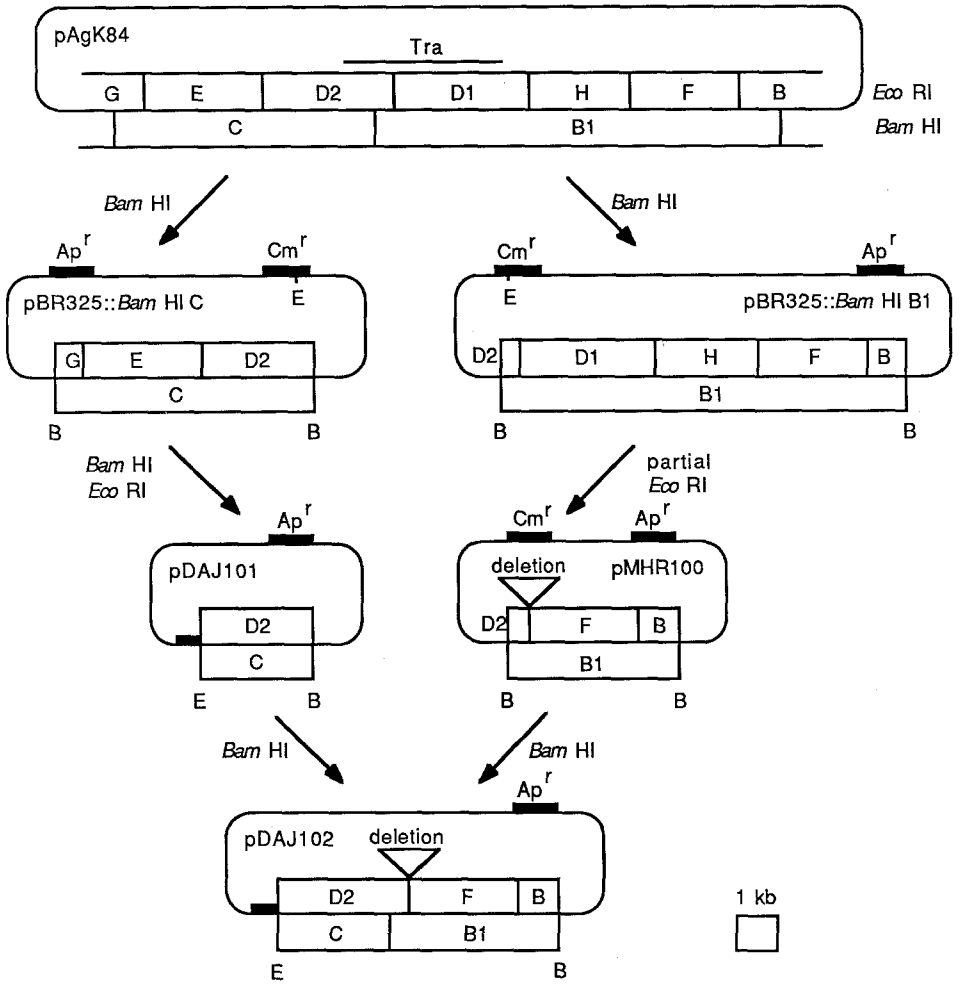

Fig. 2. Steps in the construction of intermediate plasmid pDAJ102 containing a deletion overlapping the Tra region. B and E symbolize *Bam*HI and *Eco*RI ends of the inserts, respectively. E is also used to indicate the *Eco*RI site in the Cm^r gene

or Petit's media, which were solidified with Oxoid purified-agar.

Bacterial matings. An *E. coli* helper strain (RK2013) containing the mobilizing plasmid pRK2013 (Ditta et al. 1980) was used to mobilize pBR325 constructs from *E. coli* to *Agrobacterium*. Triparental matings were carried out by mixing 1 ml of overnight liquid YEB cultures of the donor, helper and recipient, and filtering the mixtures onto 0.45 µm pore size Millipore filters. The filters were incubated on non-selective YEB agar plates overnight at 28° C. The bacteria were resuspended in 10 ml buffered saline (BS) (Shim et al. 1987), a tenfold dilution series prepared and 100-µl aliquots of appropriate dilutions plated on selective media. Conjugation was also used to transfer Tra^+ derivatives of pAgK84 between biovar 1 and 2 *Agrobacterium* strains. Biparental matings were set up as above, except that no helper strain was used and the matings were incubated for 48 h on Bergersen's medium with 0.2% sodium tartrate.

To obtain a semi-quantitative measurement of plasmid transfer ability, a droplet mating technique was used. Donors and recipient were sub-cultured for 24 h on YMA slopes and suspended in BS. Several 5-µl droplets of the recipient were placed on Petit's medium containing nopaline. The droplets were allowed to dry and then overlayed with 5-µl droplets of donors. Suitable controls consisting of recipient alone and donors alone were used. After 48 h bacteria were suspended in BS, diluted in a tenfold dilution series and 10-µl aliquots of appropriate dilutions plated on selective media to measure numbers of donors, recipients and transconjugants.

Recombinant DNA techniques. Large-scale preparation and cesium chloride density gradient purification of plasmids from *E. coli* were carried out according to Maniatis et al. (1982) and from *Agrobacterium* according to Casse et al. (1979) and Farrand et al. (1981). Mini-preparations of plasmids from *E. coli* and *Agrobacterium* were performed as described by Maniatis et al. (1982) and Farrand et al. (1985), respectively.

Restriction endonuclease digests were carried out according to the manufacturers' instructions and the conditions for partial digests were worked out as described by Maniatis et al. (1982). Restriction fragments were separated by agarose gel electrophoresis according to Maniatis et al. (1982) and recovered as described by Dretzen et al. (1981). Treatments with calf intestinal phosphatase and ligations with T4 DNA ligase were carried out according to Maniatis et al. (1982). Competent cells of *E. coli* HB101 were prepared and transformed either by the $CaCl_2$ method described by Maniatis et al. (1982) or by protocol 3 of Hanahan (1985) for the preparation and use of frozen competent cells. To identify recombinant plasmids, transformants were analysed by restriction endonuclease digestion and agarose gel electrophoresis of plasmid mini-preparations, as described above.

Agrocin 84 bioassays. The method of Stonier (1960), as modified by Kerr and Htay (1974), was used, with strain K198, which contains a nopaline + agrocinopine Ti plasmid, as the indicator.

Results

Construction of a deletion in pAgK84

A library of pAgK84 was prepared by ligating *Bam*HI digested fragments of pAgK84 into pBR325 and transforming into *E. coli* HB101. Transformants were recovered by selection on LB agar for resistance to 25 μg/ml chloramphenicol (Cm), and recombinants detected by screening on LB agar for sensitivity to 10 μg/ml tetracycline (Tc), since cloning into the *Bam*HI site of pBR325 inactivates the Tc^r gene. Two clones, pBR325::*Bam*HI B1 (in strain K840) and pBR325::*Bam*HI C (in strain K1008) (Fig. 2), which overlap the Tra region of pAgK84, were identified by analysis of single and double digests using *Bam*HI, *Eco*RI and *Sma*I (Fig. 3).

To generate a deletion, 4 μg of pBR325::*Bam*HI B1 DNA was partially digested for 1 h with 1 unit of *Eco*RI, and after checking the digestion by agarose gel electrophoresis, 1.5 μg was religated, from which 0.15 μg was used for transformation of *E. coli* HB101. Transformants were selected on LB agar for resistance to 25 μg/ml Cm, to ensure that deletions extending to the *Eco*RI site of pBR325, which is in the Cm^r gene, were not recovered. Transformants were screened for the loss of *Eco*RI fragments by analysis of *Eco*RI digests. One deletion derivative, pMHR100 (in strain K1007) (Fig. 2), lacked the contiguous *Eco*RI fragments D1 and H, a total of 5.9 kb. On one side of the deletion, *Eco*RI fragment F and the part of *Eco*RI fragment B contained within *Bam*HI fragment B1, a total of 3.7 kb, were retained. On the other side, the part of *Eco*RI fragment D2 contained within *Bam*HI fragment B1, 0.5 kb, was retained. This was confirmed by analysis of single and double digests using *Bam*HI, *Eco*RI and *Sma*I (Fig. 3).

The 0.5-kb portion of *Eco*RI fragment D2 proved insufficient to allow deletion-marker exchange by homologous recombination, so it was increased to 3.3 kb by adding the remainder of *Eco*RI fragment D2 from pBR325::*Bam*HI C, as follows: pBR325::*Bam*HI C was cut with *Bam*HI and *Eco*RI to generate five fragments, which were separated by agarose gel electrophoresis (Fig. 3). The 4.4-kb fragment, which contains the majority of pBR325 i.e. from the *Bam*HI site in the Tc^r gene to the *Eco*RI site in the Cm^r gene, and the 2.8-kb fragment, which was the part of *Eco*RI fragment D2 contained within *Bam*HI fragment C, were recovered from the gel. The 4.4-kb fragment was treated with phosphatase, ligated to the 2.8-kb fragment and transformed into *E. coli* HB101. Transformants were selected on LB agar for resistance to 40 μg/ml ampicillin (Ap) and the identity of the resultant plasmid, pDAJ101 (in strain K1022) (Fig. 2), was confirmed by analysis of single and double digests using *Bam*HI and *Eco*RI (Fig. 3).

pMHR100 was then cut with BamHI to generate two fragments, which were separated by agarose gel electrophoresis. The 4.2-kb *Bam*HI fragment B1 bearing the deletion, was recovered from the gel, ligated to pDAJ101 which had been cut with *Bam*HI and treated with phosphatase, and transformed into HB101. Transformants were selected on LB agar for resistance to 40 μg/ml Ap and the orientation of the *Bam*HI fragment B1 insert was determined by analysis of *Eco*RI digests. This generated pDAJ102 (in strain K1023) (Fig. 2) which, having reconstituted the *Eco*RI fragment D2, carried sufficient DNA on either side of the deletion to allow deletion-marker exchange by homologous recombination.

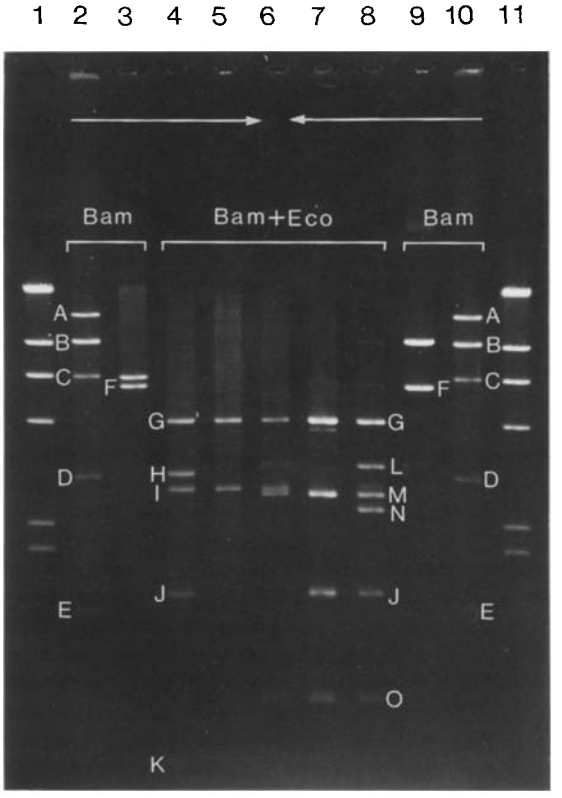

Fig. 3. *Bam*HI digests (*Bam*) and *Bam*HI and *Eco*RI double digests (*Bam* + *Eco*) of plasmids involved in the construction of intermediate plasmid pDAJ102. Fragments were separated by electrophoresis for 3 h at 100 V in a 0.7% agarose gel. Lanes 1 and 11 contain lambda phage DNA digested with *Hin*dIII; lanes 2 and 10, pAgK84 from strain K84; lanes 3 and 4, pBR325::*Bam*HI C from strain K1008; lane 5, pDAJ101 from strain K1022; lane 6, pDAJ102 from strain K1023; lane 7, pMHR100 from strain K1007; lanes 8 and 9, pBR325::*Bam*HI B1 from strain K840. The *left* and *right arrows* converging on lane 6 indicate the sequence of intermediate plasmids corresponding to the left and right pathways of Fig. 2. Band A contains *Bam*HI fragment A of pAgK84; band B, *Bam*HI fragments B1 and B2 of pAgK84 in lanes 2 and 10 but only B1 in lane 9; bands C–E, *Bam*HI fragments C–E of pAgK84; band F, pBR325; band G, the large *Bam*HI – *Eco*RI fragment of pBR325; band H, *Eco*RI fragment E of pAgK84; band I, the part of *Eco*RI fragment D2 contained within *Bam*HI fragment C of pAgK84; band J, the small *Bam*HI – *Eco*RI fragment of pBR325; band K, the part of *Eco*RI fragment G contained within *Bam*HI fragment C of pAgK84; band L, *Eco*RI fragment D1 of pAgK84; band M, *Eco*RI fragment F of pAgK84; band N, *Eco*RI fragment H of pAgK84; band O, the part of *Eco*RI fragment B contained within *Bam*HI fragment B1 of pAgK84. The part of *Eco*RI fragment D2 contained within *Bam*HI fragment B1 of pAgK84 which should be present as a very small fragment in lanes 6, 7 and 8 is not visible in this gel

pDAJ102 was mobilized from *E. coli* into *Agrobacterium* strain A28 carrying pAgK84::Tn*5*A28 by triparental mating. pDAJ102, like pBR325 its progenitor, is unable to replicate in *Agrobacterium* so that it can only confer carbenicillin (Cb) resistance if it is rescued as a cointegrate

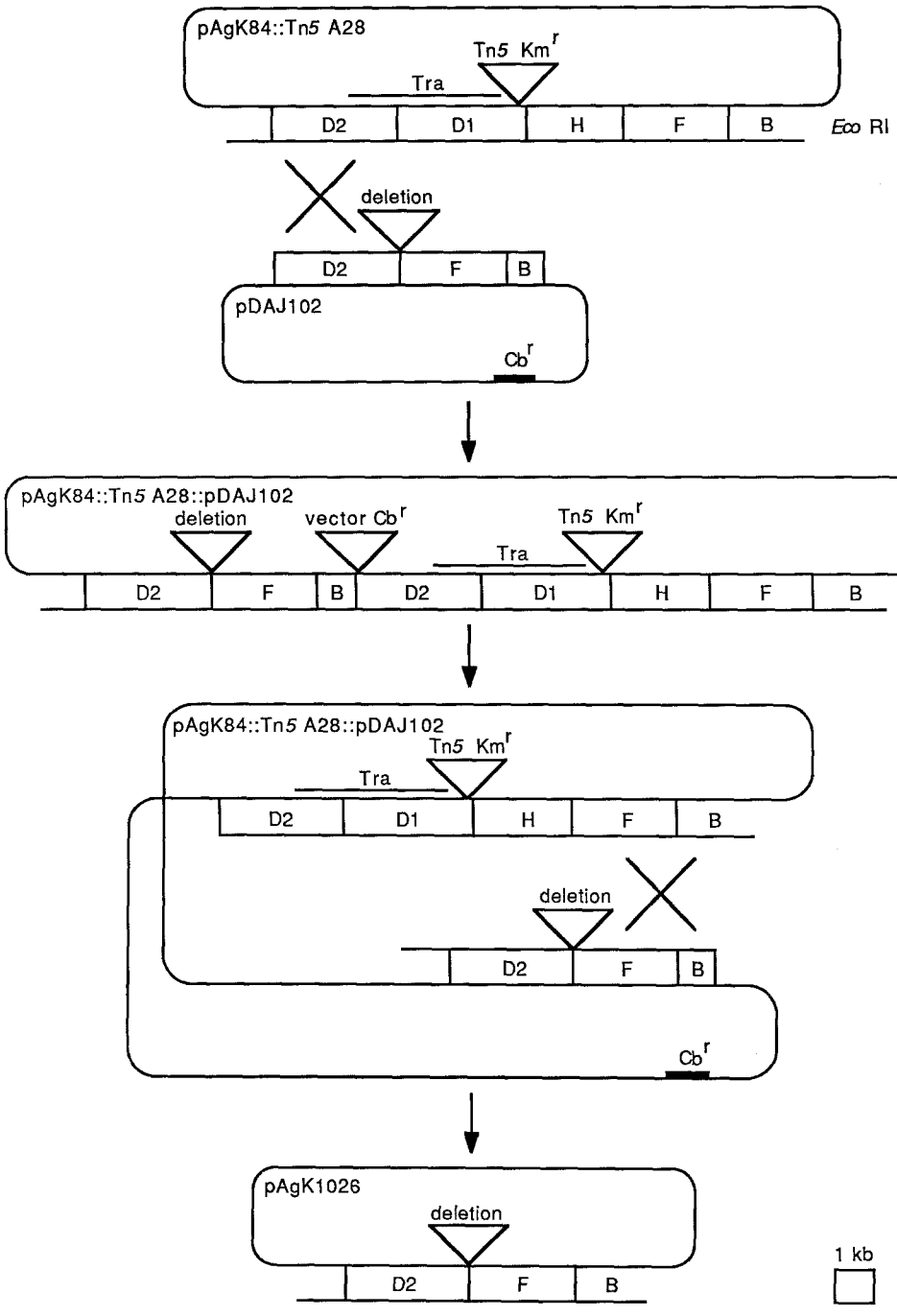

Fig. 4. Construction of pAgK1026. For illustrative purposes the homologous recombination events leading to deletion-marker exchange are shown occurring firstly in *Eco*RI fragment D2, to form a cointegrate, and then later in *Eco*RI fragment B or F, to resolve it, but they could also have occurred in the reverse order

with pAgK84 by homologous recombination. Strain K1024, a transconjugant bearing the cointegrate pAgK84::Tn*5*A28::pDAJ102 (Fig. 4), was recovered on YMA, on which the *E. coli* donor and helper strains cannot grow, by selection for resistance to 100 μg/ml Cb and 50 μg/ml kanamycin (Km), and examined for the presence of the cointegrate by analysis of *Eco*RI digests (Fig. 5). The A28 Tn*5* insertion lies inside the region covered by the deletion but just outside the Tra region (Farrand et al. 1985) (Fig. 4), so the cointegrate was Tra^+.

The cointegrate was conjugatively transferred to *Agrobacterium* strain K434 in a biparental mating. Strain K434, a derivative of strain K84 lacking pAgK84 but otherwise retaining the plasmid complement (Fig. 6) and chromosomal background of its progenitor, was chosen as the recipient so that the modified agrocin plasmid would be placed back in its original genetic environment. Strain K1025, a transconjugant containing the cointegrate, was recovered on Bergersen's medium containing 0.2% sodium tartrate as the sole carbon source (on which the biovar 2 recipient could grow but the biovar 1 donor could not) by selection for resistance to 200 μg/ml Km. It was checked for the presence of the cointegrate by analysis of undigested and *Eco*RI digested plasmid mini-preparations (Fig. 5). To allow the second recombination event forming the deletion-marker exchange (Fig. 4), strain K1025 was grown non-selectively for three subcultures, each time inoculating 100 μl of a saturated 24-h liquid YEB culture into 10 ml of fresh liquid YEB, prior to plating on YEB agar at a colony density of ca. 150–200 colonies/plate. The resultant colonies were

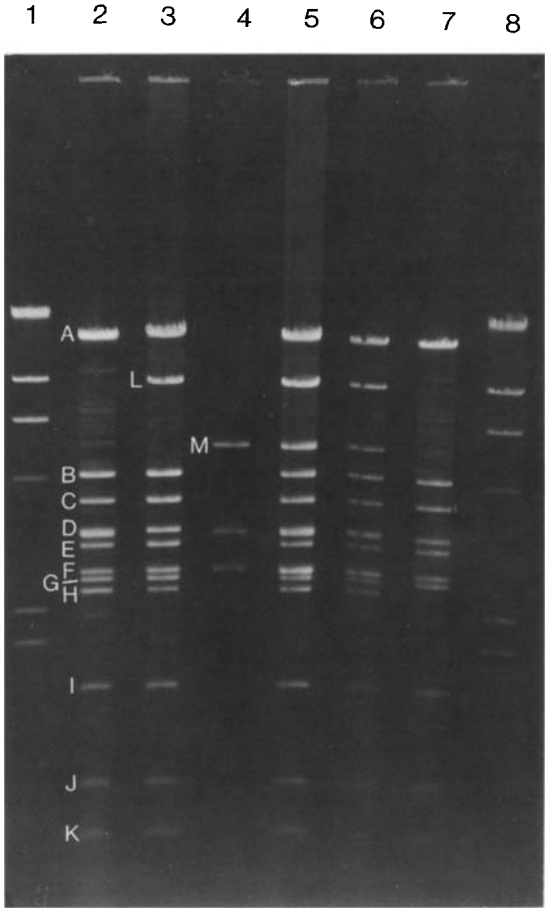

Fig. 5. *Eco*RI digests of plasmids involved in the construction of pAgK1026. Fragments were separated by electrophoresis for 3 h at 100 V in a 0.7% agarose gel. Lanes 1 and 8 contain lambda phage DNA digested with *Hin*dIII; lane 2, pAgK84 from strain K84; lane 3, pAgK84::Tn*5*A28 from strain A28; lane 4, pDAJ102 from strain K1023; lane 5, pAgK84::Tn*5*A28::pDAJ102 from strain K1024; lane 6, pAgK84::Tn*5*A28::pDAJ102 from strain K1025; lane 7, pAgK1026 from strain K1026. Lanes 2, 6, 7 also contain a background of restriction fragments derived from pAtK84b. Bands A–C contain *Eco*RI fragments A–C of pAgK84; band D, *Eco*RI fragments D1 and D2 of pAgK84 in lane 2 but only D2 in lanes 3–7; bands E–K, *Eco*RI fragments E–K of pAgK84; band L, *Eco*RI fragment D1 of pAgK84 containing Tn*5* which has no *Eco*RI sites; band M, an *Eco*RI fragment containing the large *Eco*RI – *Bam*HI fragment of pBR325 joined via a *Bam*HI site to the part of *Eco*RI fragment B contained within *Bam*HI fragment B1 of pAgK84

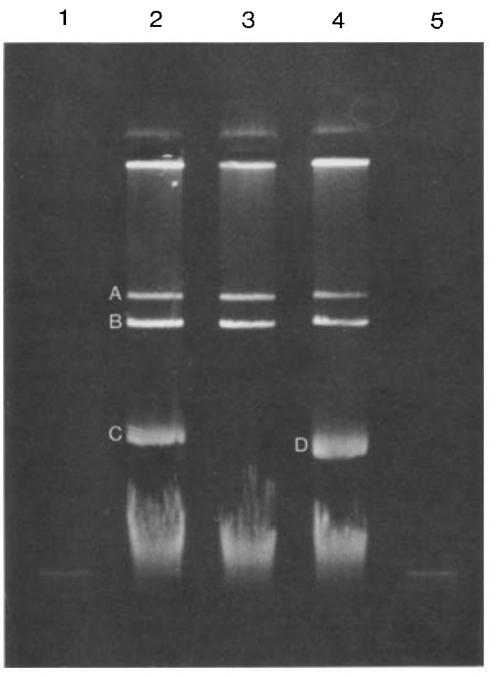

Fig. 6. Plasmids contained within *Agrobacterium* strains K84, K434 and K1026. Undigested plasmids were separated by electrophoresis for 3 h at 100 V in a 0.7% agarose gel. Lanes 1 and 5 contain undigested lambda phage DNA; lane 2, plasmids from strain K84; lane 3, plasmids from strain K434; lane 4, plasmids from strain K1026. Band A contains the cryptic plasmid; band B, pAtK84b, the nopaline catabolizing plasmid; band C, pAgK84; band D, pAgK1026

replica plated onto YEB agar and YEB agar containing 200 μg/ml Km. Strain K1026, a spontaneous kanamycin-sensitive derivative of strain K1025, which had resolved the cointegrate by homologous recombination to generate a deletion-marker exchange (Fig. 4), was recovered as a single occurrence among about 7000 colonies replicated. The identity of the mutant plasmid, designated pAgK1026, in strain K1026 was confirmed by analysis of *Eco*RI digested (Fig. 5) and undigested (Fig. 6) plasmid mini-preparations. The latter also confirmed retention of the plasmid complement of strain K84 by strain K1026.

The complete loss of vector and Tn*5* sequences from strain K1026 may be inferred by the loss of Cb resistance carried by the vector and Km and streptomycin (Sm) resistance carried by Tn*5*. This indicates that no foreign DNA remains in strain K1026.

Properties of pAgK1026

Strains K84 and K1026 were tested for agrocin 84 production using equivalent cell numbers of the two strains in order to semi-quantify the amount of agrocin produced. Strain K1026 produces agrocin 84 (Fig. 7), indicating that pAgK1026 retains the agrocin 84 biosynthetic capacity of its pAgK84 progenitor. Furthermore, the sizes of the inhibition zones for strains K84 and K1026 were similar (Fig. 7), indicating that they produce similar amounts of agrocin 84. This provides indirect evidence that pAgK1026 retains the copy number of its pAgK84 progenitor, since Shim et al. (1987) found that a mutant of pAgK84 with increased copy number produced a correspondingly increased amount of agrocin 84.

To study plasmid stability and transfer ability, both pAgK84 and pAgK1026 were marked with the Cm^r and Cb^r genes of pBR325, as follows. pBR325::*Bam*HI C was transferred by triparental mating from strain K1008 to both strains K1026 and K84, where pBR325::*Bam*HI C formed a cointegrate with pAgK1026 and pAgK84, respectively, by homologous recombination. Transconjugant strains K1027, containing pAgK1026::pBR325::*Bam*HI C, and K1028, containing pAgK84::pBR325::*Bam*HI C, were recovered on YMA, on which the *E. coli* donor and helper strains could not grow, by selection for resistance to 100 μg/ml Cm and 500 μg/ml Cb, and checked for the presence of their respective cointegrates by analysis of *Eco*RI digests (data not shown).

To assay for plasmid stability, strains K1027 and K1028 were subcultured non-selectively ten times, as described above, prior to plating on YEB agar at a colony density

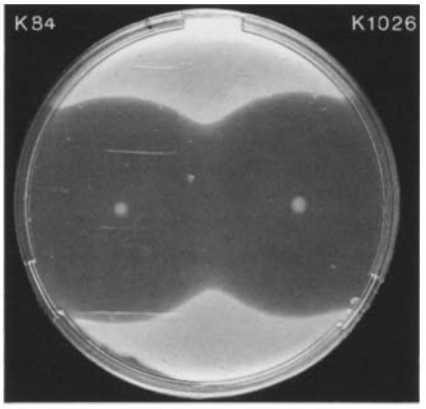

Fig. 7. Bioassay for the production of agrocin 84 by *Agrobacterium* strains K84 and K1026. Agrocin production is indicated by the zones of inhibition in the growth of the strain K198 overlay on Stonier's medium

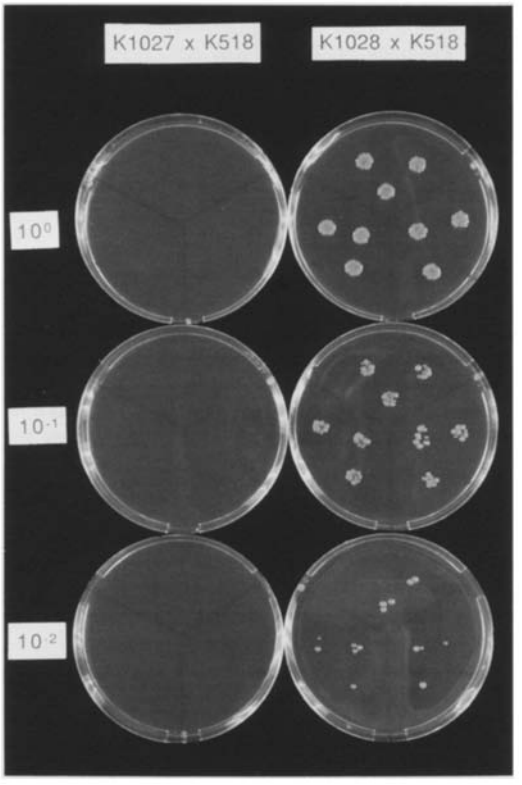

Fig. 8. Transfer ability of pAgK84 and its engineered deletion derivative, pAgK1026. Strain K1027, containing pAgK1026, and strain K1028, containing pAgK84, were mated with strain K518 and 10 µl droplets of a tenfold dilution series of the mating mixture were spotted onto media selecting for transconjugants, as described in Materials and methods

of ca. 40 colonies/plate. The resultant colonies were replica plated onto YEB agar and YEB agar containing 100 µg/ml Cm and 500 µg/ml Cb. For strain K1027, 28 colonies sensitive to Cm and/or Cb were recovered out of 1412 replica plated, giving 1.98% marker loss after ten subcultures. Similarly for strain K1028, 35 colonies sensitive to Cm and/or Cb were recovered out of 1834 replica plated, giving 1.91% marker loss after ten subcultures, which is not significantly different to that for strain K1027 ($P > 0.87$). The antibiotic sensitive isolates were then assayed for agrocin 84 production and all were found to produce the antibiotic, indicating that they had not lost their respective agrocin plasmids. They presumably lost their markers by resolution of the cointegrate, with the concomitant loss of pBR325::*Bam*HI C which cannot replicate independently in *Agrobacterium*. Thus, there was no loss of pAgK1026 or pAgK84 after 10 subcultures, indicating that pAgK1026 retains the stability of its progenitor pAgK84.

To assay for plasmid transfer ability, strains K1027 and K1028 were crossed to K518 in biparental droplet matings on Petit's agar containing 0.2% nopaline, as described in Materials and methods. Donor and recipient strains were able to grow on the mating medium because each harbours pAtK84b, a nopaline catabolizing plasmid. Numbers of donors were determined on NA containing 50 µg/ml Cm, numbers of recipients on NA containing 50 µg/ml rifampicin (Rif), and numbers of transconjugants on NA containing 50 µg/ml Rif, 50 µg/ml Cm and 100 µg/ml Cb. No transconjugants were observed from the cross in which K1027 was the donor (Fig. 8). This gave transmission frequencies of $< 3.84 \times 10^{-7}$ per donor and $< 1.96 \times 10^{-8}$ per recipient. When K1028 was the donor many transconjugants were observed (Fig. 8), giving transmission frequencies of 3.34×10^{-4} per donor and 3.96×10^{-5} per recipient. Clearly pAgK1026 is a Tra^- mutant of pAgK84.

Discussion

Continued success in the biological control of crown gall by *Agrobacterium* strain K84 is threatened by the transfer of pAgK84 from strain K84 to pathogenic agrobacteria (Ellis and Kerr 1979; Panagopoulos et al. 1979). To overcome this problem we have constructed pAgK1026, a deletion mutant of pAgK84, which is transfer deficient. This plasmid remains stable and agrocinogenic even though the deletion extends anticlockwise beyond the Tra region by 3.1 kb. This plasmid is carried by strain K1026, which has the same chromosomal background and plasmid complement as strain K84. The genetic background of strain K84 is important because, unlike some others, it confers the ecological competence to efficiently colonize roots which is necessary for effective control (Shim et al. 1987). Beside the agrocin plasmid, strain K84 harbours a large cryptic plasmid (Ellis et al. 1979), and a nopaline-catabolizing plasmid, pAtK84b (Sciaky et al. 1978) (Fig. 6). The role of the cryptic plasmid in biological control, if any, is unknown. The nopaline-catabolizing plasmid carries large regions of homology with various Ti plasmids, including the incompatibility region (B.G. Clare and A. Kerr, unpublished data), and is functionally Ti plasmid incompatible (S.K. Farrand, unpublished data). This incompatibility reduces the likelihood of strain K84 or K1026 becoming a pathogen by conjugative acquisition of a Ti plasmid.

Apart from its inability to transfer the agrocin plasmid by conjugation, strain K1026 is essentially the same as strain K84. So, it is potentially a suitable and desirable replacement for the latter, and should prolong the effective biological control of crown gall. However, the critical test of its suitability is whether it provides effective control in planta. To this end, we have obtained permission from the appropriate Australian authorities for the environmental release of strain K1026. The release involves open-air pot trials at the Waite Institute, in which strain K1026 is being

compared with strain K84, for its efficacy in controlling crown gall on almond seedlings.

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