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Short communication

The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumour induction

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Summary. When the 225 kilobase (kb) cryptic plasmid of *Rhizobium meliloti* 41 is introduced into *Agrobacterium tumefaciens* C58, the resident plasmid pAtC58 (410 kb) is lost, probably because of incompatibility. The strain of *A. tumefaciens* cured of pAtC58 is still oncogenic, showing that pAtC58 does not control functions essential for tumour formation in the tomato and in *Kalanchoe daigremontiana.*

Introduction

The bacterial family *Rhizobiaceae* is composed of two genera, *Agrobacterium* and *Rhizobium,* both of which are able to establish close interactions with higher plants.

Most *Agrobacterium* and fast-growing *Rhizobium* strains investigated so far harbour more than one large plasmid ranging in size from 130 kb to more than 700 kb (Zaenen et al. 1974; Nuti et al. 1977; Casse et al. 1979; Rosenberg et al. 1981; Banfalvi et al. 1981). Some of these plasmids have been shown to control directly the interactions of bacteria and plants. *A. tumefaciens,* which induces Crown gall disease, carries Ti plasmids which control virulence (Van Larebeke et al. 1974). The virulence of *A. Rhizogenes,* the causative agent of hairy root disease, is conferred by Ri plasmids (Moore et al. 1979).

In fast-growing rhizobia, genes controlling nodulation, host-range specificity and nitrogen fixation are located on large plasmids (for a review, see Dénarié et al. 1982). However, several plasmids have been observed in the *Rhizobiaceae,* the functions of which are still unknown. For instance, large cryptic plasmids are present in many strains of *A. tumefaciens* (Schell et al. 1976; Casse et al. 1979; Van Montagu and Schell 1979). One way to determine whether such plasmids are involved in plant-bacteria interactions is to test strains of *Agrobacterium* cured of these plasmids for oncogenicity. A classical procedure for eliminating plasmids from bacteria is to introduce another plasmid of the same incompatibility group into the bacterium. Only a few data are available about plasmid incompatibility in *Rhizobiaceae.* Two bacteriocinogenic plasmids of *R. leguminosarum* (Brewin et al. 1980) belong to the same incompatibility group; a plasmid controlling nodulation in *R. phaseoli* is incompatible with a small plasmid from *R. leguminosarum* (Johnston et al. 1982). Recently, Hooykaas (1984) identified four incompatibility groups. Group IncRH-1 contains pTi plasmids of octopine and nopaline strains, while pTi of neutral type strains, pRi of *A. rhizogenes* and pSym of *R. trifolii* belong to IncRh-2, IncRh-3 and IncRh-4 groups, respectively. In order to investigate the compatibility of various *Rhizobiaceae* plasmids, we transferred the 225 kb cryptic plasmid pRme41 of *R. meliloti* strain 41 to other *R. meliloti* strains and also to *R. trifolii* and *A. tumefaciens* strains. In this paper, we report that the introduction of pRme41 into *A. tumefaciens* C58 causes the loss of an indigenous cryptic plasmid of 410 kb (Casse et al. 1979), suggesting that these two plasmids belong to the same incompatibility group. The curing of pAtC58 does not result in loss of oncogenicity in the tomato or in *Kalanchoe.*

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Results and discussion

A plasmid from Rhizobium and a plasmid from Agrobacterium belong to the same incompatibility group

Since no genetic marker was available to select for the transfer of pRme41, the plasmid was tagged with Tn*5* (conferring resistance to Km and Nm) using the technique of region-specific mutagenesis (Huguet et al. 1983). One of these pRme41::Tn*5* plasmids, pGMI59, was transferred into GMI9027, a Rif^r Sm^r Sp^r derivative of *A. tumefaciens* C58. Plasmid pGMI59 was shown to be self-transmissible within *R. meliloti* strains 41 and L5-30 (frequency of about 10^{-6} per initial donor), but was not self-transmissible to GMI9027 at a detectable frequency. To mobilize pGMI59 we used an RP4-prime plasmid, pGMI4142, which consists of a 45 kb fragment of pRme41 cloned in the *Hin*dIII site of the broad host-range plasmid RP4 (Huguet et al. 1983). The results of the mating are shown in Table 2, mating 1. The plasmid profile of *Agrobacterium* transconjugants was examined on agarose gels. Figure 1 shows that in one representative transconjugant, GMI9008, the upper band corresponding to pAtC58 in *A. tumefaciens* strain GMI9027 (lane B) was not visible in the transconjugant strain GMI9008 (lane C), while bands corresponding to pGMI59, pTi and the mobilizing plasmid pGMI4142 were present. The loss of the pAtC58 band seems to be due to the presence of pGMI59 and not of pGMI4142 since the transfer of pGMI4142 alone did not result in the loss of the pAtC58 band (data not shown).

In order to determine whether the pAtC58 plasmid plays a role in the pathogenicity of *A. tumefaciens* C58, it was necessary to cure the strain GMI9008 of pGMI59 and

Table 1. Bacteria, bacteriophages and plasmids used

Strains	Relevant characteristics	Source or reference
Bacteria		
E. coli		
GMI3629	pK1046 (pGMI175)	Rosenberg et al. (in preparation)
R. meliloti		
GMI17	Rm41 (pGMI59) (pGMI4142)	Huguet et al. (1983)
A. tumefaciens		
C58		Casse et al. (1979)
C58C1		Casse et al. (1979)
GMI9027	Sm^s Rif^r Sp^r derivative of C58	This work
GMI9008	GMI9027 (pGMI59) (pGMI4142) cured of pAtC58	This work
GMI9022	GMI9027 (pGMI4142) cured of pAtC58	This work
GMI9033	GMI9027 (pGMI175) cured of pAtC58	This work
GMI9017	GMI9027 cured of pAtC58	This work
GMI9028	GMI9027 cured of pAtC58 in which pAtC58::Tn*1* has been reintroduced	This work
GMI9023	Sm^r Rif^r derivative of C58C1 cured of pAtC58	This work
GV3350	An Em^r Cm^r derivative of C58C1 with a Tn*1* insertion (conferring resistance to Cb) in pAtC58	Laboratory of Genetics, Gent University
Bacteriophages		
S2 and S5	Specific for *A. tumefaciens*	J.P. Hernalsteens, Laboratory of Genetics, Gent University
7711	Mu tagged with the Km resistance determinant of Tn*5*	Thomson and Howe, Madison University
Plasmids		
RP4	Tc^r, Km^r, Cb^r	Datta et al. (1971)
pGMI59	pRme41 tagged with Tn*5* (insertion number 4)	Huguet et al. (1983)
pGMI4142	RP4 carrying a 45 kb pRme41 fragment cloned in the *Hin*dIII site	Huguet et al. (1983)
pRP301	Km^s derivative of RP4	Barth (1979)
pGMI175	pRP301 carrying a 7711 prophage	Rosenberg et al. (in preparation)

Antibiotics: Cb, carbenicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Nm, neomycin; Rif, rifampicin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline

pGMI4142 prior to testing it for oncogenicity, since exogenous plasmids could interfere with tumour production (Farrand et al. 1981), and to check that the absence of the upper band in lysates of strain GMI9008 actually resulted from the loss of pAtC58.

Since it has been shown that heat treatment can affect plasmid stability in *A. tumefaciens* (Van Larebeke et al. 1974), a similar instability of pGMI59 was investigated. For this purpose a culture of GMI9008 grown at an elevated temperature (34° C), in the absence of antibiotics, was spread onto non-selective agar plates. After 48 h at 34° C, the plates were replicated onto medium supplemented with 30 µg/ml of kanamycin. Of 3×10^3 colonies tested one was Km^s indicating the loss of Tn*5*.

Table 2. Bacterial crosses

Mating number	Donor	Recipient	Transconjugant frequency
1	GMI17	GMI9027	3×10^{-7}
2	GMI3629	GMI9022	10^{-4}
3	GV3350	GMI9017	10^{-7}

The matings were performed as described (Truchet et al. 1984). Transconjugant frequencies are reported per initial donor. Selection was in the presence of Km (30 µg/ml in matings 1 and 2, and Cb (200 µg/ml) in mating 3. Counterselection of the donors was in the presence of Sp (100 µg/ml) and Rif (100 µg/ml) in mating 1, Sm (250 µg/ml) in mating 2, and Rif (100 µg/ml) and Cm (25 µg/ml) in mating 3. Transconjugants of matings 1 and 2 were tested for sensitivity to *A. tumefaciens*-specific phages S2 and S5. Transconjugants of mating 3 were tested for resistance to Sp and sensitivity to Er

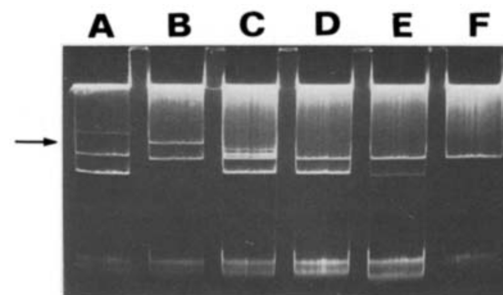

Fig. 1. Agarose gel electrophoresis of lysates from *R. meliloti* GMI17 (A), carrying in addition to the megaplasmids, pGMI59 and pGMI4142, and *A. tumefaciens* GMI9027 carrying pAtC58 (arrow) and pTi (B), GMI9008 (C), GMI9022 (D), GMI9033 (E), GMI9017 (F). Lysis and electrophoresis were performed according to Rosenberg et al. (1982)

Agarose gel electrophoresis of a lysate of the Km^s strain, referred to as GMI9022, showed that the band corresponding to pGMI59 had disappeared, while the pTi and pGMI4142 bands were still present (Fig. 1, lane D).

To cure GMI9022 of pGMI4142, we applied a general method for curing *Rhizobiaceae* of IncP-1 plasmids which will be described in detail elsewhere (Rosenberg et al., in preparation). Firstly, the IncP-1 resident plasmid was chased from the strain by introducing a Km^s derivative of RP4, pRP301, carrying a bacteriophage Mu::Km^r (phage 7711). This hybrid plasmid pRP301::7711 (=pGMI175) did not show any "suicide" effect when transferred to *Rhizobiaceae*, but the transconjugants grew slowly. One of these transconjugants was referred to as GMI9033 (see Table 2, mating 2, and Fig. 1, lane E). Secondly, by taking advantage of the "petite" colony phenotype conferred by the presence of the prophage, bacteria which have lost pGMI175 could be easily obtained by streaking GMI9033 onto non-selective medium, and isolating the large-colony segregants (Fig. 1, lane F). One of these

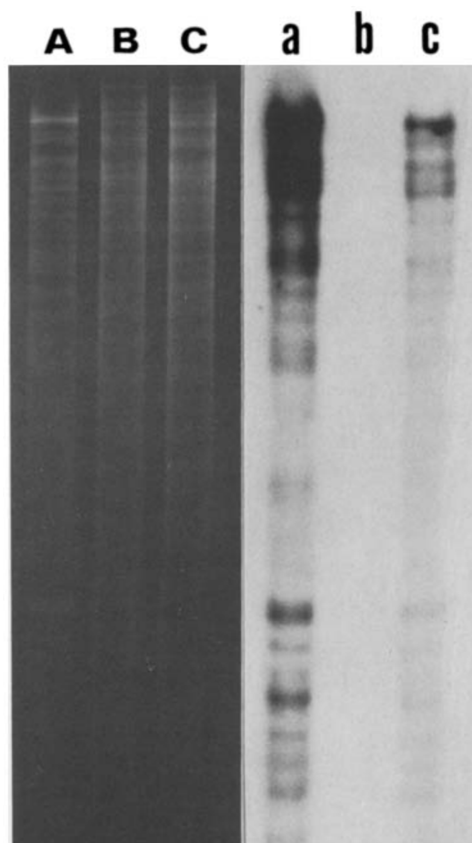

Fig. 2. Hybridization experiments. Lane A shows a *Hin*dIII digest of plasmid pAtC58. Lanes B and C show a *Hin*dIII digest of total DNA from strains GMI9017 and GMI9027 respectively. Lanes a, b and c show autoradiographs of transfers of lanes A, B and C hybridized with a ^{32}P-labelled pAtC58 probe. Total DNA was extracted by a lysozyme and sarcosyl treatment followed by banding in a CsCl/EtBr gradient. Plasmid pAtC58 was purified according to Rosenberg et al. (1982)

segregants, referred to as GMI9017, was used to check for the actual loss of pAtC58 by hybridization experiments.

Total DNA digested with *Hin*dIII from strains GMI9017 and GMI9027 and *Hin*dIII-digested pATC58 plsmid DNA was run on a Tris-acetate agarose gel, blotted onto a nitrocellulose sheet, and hybridized with a pAtC58 probe labelled with ^{32}P by nick translation (Fig. 2). No hybridization was detected with GMI9017 total DNA, indicating that GMI9017 was actually cured of pATC58.

Plasmid pATC58 was reintroduced into GMI9017. Using the strain GV3350, which is a derivative of C58C1 harbouring a pATC58 plasmid tagged with Tn*1* (conferring resistance to carbenicillin), Van Montagu and Schell (1979) have shown that pAtC58 is self-transmissible to other *Agrobacterium* strains. This strain GV3350 was used to reintroduce pAtC58 into GMI9017 (see Table 2, mating 3). The presence of pAtC58 in the transconjugant strain GMI9028 was checked by agarose gel electrophoresis. The fact that pAtC58 is able to replicate in GMI9017 indicates that its loss was probably due to incompatibility, and that plasmids from *Agrobacterium* and *Rhizobium* belong to the same incompatibility group.

pAtC58 is not essential for tumour induction

Plant tests were performed on two hosts, tomato (*Lycopersicon esculentum*) and *Kalanchoe daigremontiana*. Plant stems were wounded in two places with a sterile blade and inoculated with 10 µl of bacteria suspended in sterile water (3×10^9 bacteria/ml). After one month, ten out of the ten plants inoculated with GMI9017 showed tumours, as did those inoculated with strain GMI9028 containing pAt::Tn*1*. The negative controls, inoculated with C58C1 did not show any tumour. We conclude that the pAtC58 plasmid does not control functions essential for oncogenicity under our assay conditions. Preliminary studies of strain GMI9017 metabolism suggest that pAtC58 controls catabolic functions (J. Tempé, personal communication).

In addition, a derivative of C58C1 cured of pAtC58, GMI9023, has been obtained in a similar way. Such a *Rhizobiaceae* strain completely devoid of plasmids can be useful as a recipient for plasmid transfer experiments: it avoids potential interactions between resident plasmids and pTi, pRi or pSym *Rhizobium* plasmids transferred to an *Agrobacterium* host (Truchet et al. 1984). This recipient strain could also be used for the purification of large *Rhizobiaceae* plasmids which is usually difficult because of the presence of several of these plasmids in the original strains.

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