Introduction of Bacteriophage Mu into *Pseudomonas solanacearum* and *Rhizobium meliloti* using the R Factor RP4

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SUMMARY

Phage Mu-1 and a thermoinducible derivative, Mu-1 *cts* 62 were inserted into the broad host range R factor RP4. These hybrid plasmids were transferred by conjugation to a phytopathogenic bacterium *Pseudomonas solanacearum* GM1000 and a legume-root nodule bacterium *Rhizobium meliloti* 2011. The Mu genome is transcribed and translated in these new hosts: *P. solanacearum* (RP4::Mu *cts*) cultures have a spontaneous production of about 5×10^5 plaque-forming units ml⁻¹ which is similar to the frequency of spontaneous Mu production in *E. coli*; the Mu production of *R. meliloti* is lower (about 10^2 plaque-forming units ml⁻¹).

INTRODUCTION

The temperate bacteriophage Mu can integrate at random into the chromosome of its host *Escherichia coli* K12 (Taylor, 1963; Bukhari & Zipser, 1972; Daniell, Roberts & Abelson, 1972). Insertion into a gene can make it inactive and give rise to a mutation (Taylor, 1963). Mu can mediate deletion (Howe & Bade, 1975) and transposition of its host DNA (Faellen & Toussaint, 1976) and is also able to connect two unrelated DNA sequences. After infection or induction, it can promote integration of circular DNA, such as λ *gal* (Faellen, Toussaint & Couturier, 1971; Toussaint & Faellen, 1973) or F (Van de Putte & Gruijthuijsen, 1972), into the *E. coli* chromosome. Using Mu, F and F' factors have been integrated into the chromosome of *recA* *E. coli* strains giving rise to Hfr strains which have different origins and orientations (Van de Putte & Gruijthuijsen, 1972). This technique has been used to generate Hfr strains in Mu-sensitive strains of *Citrobacter freundii*, another member of the Enterobacteriaceae (de Graaf, Kreuning & Van de Putte, 1973).

Unfortunately the host range of Mu is limited to only a few of the Enterobacteriaceae: *E. coli* K12, *Shigella dysenteriae* (Taylor, 1963) and *C. freundii* (de Graaf *et al.*, 1973). It would be useful to have a method for extending the host range of this phage. When inserted into the F sex factor, Mu has been transferred by conjugation between *E. coli* K12 derivatives (Schröder & Van de Putte, 1974; Razzaki & Bukhari, 1975). But the host range of F is also limited to enterobacteria closely related to *E. coli*. RP4 is an R factor which originated from *Pseudomonas aeruginosa* (Datta *et al.*, 1971). Like other plasmids of the P incompatibility group, it has a very broad host range among Gram-negative bacteria (Datta & Hedges 1972; Olsen & Shipley, 1973). It has been transferred to nitrogen-fixing bacteria such as *Rhizobium* (Datta & Hedges, 1972; Beringer, 1974) and *Azotobacter* (Olsen & Shipley, 1973) and to numerous phytopathogenic bacteria (Message, Boucher & Boistard, 1975; Panopoulos *et al.*, 1975). Therefore RP4 might be a useful vector for Mu. This investiga-

tion was carried out in an attempt to answer two questions: (i) when inserted into RP4 can the Mu prophage be introduced into *Pseudomonas solanacearum* and *Rhizobium meliloti*, which are taxonomically distant from the enteric bacteria; and (ii) can the Mu genome be expressed in these new hosts and does this expression lead to the production of plaque-forming particles?

METHODS

Bacterial and bacteriophage strains. These are listed in Table 1. Genetic nomenclature of the bacteria follows the proposals of Demerec *et al.* (1966). Phage Mu-1 is referred to as Mu and the Mu-1 *cts* 62 mutant as Mu *cts*. The symbol :: indicates that Mu is inserted in the plasmid preceding the symbol (Howe & Bade, 1975), for example, RP4::Mu *cts* means that the thermoinducible prophage is inserted in the RP4 plasmid.

Media and growth conditions. *Escherichia coli* was grown in L broth (Lennox, 1955) or in M63 minimal medium (Monod, Cohen-Bazire & Cohn, 1951). *Pseudomonas solanacearum* was grown on TZC agar medium (Kelman, 1954) supplemented with Difco yeast extract (1 g l⁻¹) (BGT medium). Liquid cultures were obtained in BG broth containing (g l⁻¹): Difco Bacto-peptone, 10; Difco Casamino acids, 1; Difco yeast extract, 1; glucose, 5. The minimal medium (MM) described by Abo-El-Dahab & El Gorani (1969) was supplemented with glucose (5 g l⁻¹). The complex (C) and minimal (V) media used for *R. meliloti* were those described by Vincent (1970), modified as follows: C was supplemented to contain 0.1 % (w/v) Casamino acids (Difco) and 0.15 % (w/v) yeast extract (Difco); V was supplemented with KNO₃ (0.6 g l⁻¹) and biotin (250 µg l⁻¹). Solid media were prepared by adding purified Difco Bacto-agar (15 g l⁻¹). Bacterial dilutions were made in saline for *E. coli* and in distilled water for *R. meliloti* and *P. solanacearum*. All the strains were grown at 28 °C unless otherwise stated.

For Mu phage production, dilution and titration, L medium was supplemented with 0.1 mM-L-tryptophan, 2.5 mM-CaCl₂ and 1 mM-MgSO₄ (Taylor, 1963). The phage Π1 was produced and tested on the bacteriophage medium described by Vincent (1970). The *a* phage sensitivity tests were performed on BG medium.

Bacterial matings. When quantitative results were not wanted, for instance in obtaining new R⁺ strains, spot matings were used. The recipient strain was spread on the surface of a selective agar plate and the R⁺ donors were added in small drops. The R⁺ transconjugants were then purified three times by streaking for single colonies on selective medium.

To determine transfer frequencies, matings were performed on filter membranes (de Graaf *et al.*, 1973). Crosses between *E. coli* and *P. solanacearum* were made as follows. Exponentially growing R⁺ donors at approximately 2 × 10⁸ colony-forming units (c.f.u.) ml⁻¹ were mixed with an equal volume of recipient cultures in the late exponential phase (about 10⁹ c.f.u. ml⁻¹). The mixture was filtered on a Gelman filter membrane (pore size 0.45 µm, φ 25 mm). Membranes were placed on the surface of a BG agar plate and incubated at 28 °C for 3 h. Bacteria were then resuspended in 5 ml of the dilution medium of the recipient and 0.1 ml of suitable dilutions was spread on the selective medium. For crosses between *E. coli* and *R. meliloti*, the donor:recipient ratio was 1:1; membranes were laid on agar plates and incubated at 28 °C for 5 h except when stated otherwise. For heat treatment of *R. meliloti* recipient, cultures in complete medium were shifted to 47 °C for 6 min, and then immediately mixed with the donor culture and filtered. Donors were counter-selected either by addition of streptomycin (Str) (500 µg ml⁻¹ for *E. coli*, 200 µg ml⁻¹ for *P. solanacearum* and *R. meliloti*) or by omission of nutritional requirements. Transfer of RP4 was selected with tetracycline (Tet) (10 µg ml⁻¹) or kanamycin (Kan) (25 µg ml⁻¹ for

Table 1. *Bacterial strains, phages and plasmids*

Bacteria	Relevant characteristics*	Reference or source†
<i>E. coli</i>		
K12S	Wild-type	R. Devoret
K12S (Mu <i>cts</i>)		Lysogenized with Mu <i>cts</i>
K12S (Mu <i>cts</i>) (RP4)		J53 (Mu <i>cts</i>) (RP4) × K12S (Mu <i>cts</i>)
K12S (Mu <i>cts</i>) (RP4::Mu <i>cts</i>)		J53 (Mu <i>cts</i>) (RP4::Mu <i>cts</i>) × K12S (Mu <i>cts</i>)
J53 (RP4)	Pro ⁻ Met ⁻	Clowes & Hayes (1968)
J53 (Mu <i>cts</i>) (RP4)		Lysogenized with Mu <i>cts</i>
J53 (Mu <i>cts</i>) (RP4::Mu)		Dénarié <i>et al.</i> (1976)
J53 (Mu <i>cts</i>) (RP4::Mu <i>cts</i>)		Lysogenized with Mu <i>cts</i>
AB1157	Multiple auxotroph Str-r	Howard-Flanders & Theriot (1966)
AB1157 (Mu <i>cts</i>)		Lysogenized with Mu <i>cts</i>
AB1157 (Mu <i>cts</i>) (RP4)		J53 (Mu <i>cts</i>) (RP4) × AB1157 (Mu <i>cts</i>)
AB1157 (Mu <i>cts</i>) (RP4::Mu)		J53 (Mu <i>cts</i>) (RP4::Mu) × AB1157 (Mu <i>cts</i>)
AB1157 (Mu <i>cts</i>) (RP4::Mu <i>cts</i>)		J53 (Mu <i>cts</i>) (RP4::Mu <i>cts</i>) × AB1157 (Mu <i>cts</i>)
AB1157 Mu-r	Mu resistant	Resistance to Mu c 4
c600	Str-r rK ⁻ mK ⁻ Thr ⁻ Leu ⁻	Thomas, Cameron & Davis (1974)
c600 (Mu <i>cts</i>)		Lysogenized with Mu <i>cts</i>
c600 (Mu)		Lysogenized with Mu
c600 Mu-r	Mu resistant	Resistance to Mu c 4
<i>P. solanacearum</i> GM11000	Wild-type	Message <i>et al.</i> (1975)
<i>R. meliloti</i>		
2011	Wild-type	Scherrer & Dénarié (1971)
2011 Str-r	Str-r	
2011 Str-r Gly ⁻	Str-r Gly ⁻	
Phages		
Mu-1	Wild-type	Taylor (1963)
Mu-1 <i>cts</i> 62	Thermoinducible mutant	Howe (1973)
Mu c 4	Clear mutant	M. Couturier (Univ. libre de Bruxelles, Belgium)
<i>a</i>	Temperate on <i>P. solanacearum</i> GM11000	Message <i>et al.</i> (1975)
111	Virulent on <i>R. meliloti</i> 2011	M. Obaton (I.N.R.A., Dijon, France)
Plasmids‡		
RP4	Amp, Kan, Tet	Datta <i>et al.</i> (1971)
pGM12	RP4::Mu-1	Dénarié <i>et al.</i> (1976)
pGM14	RP4::Mu-1 <i>cts</i> 62	This paper

* rK⁻ mK⁻, deficient for the K system of DNA restriction and modification; Mu-r, strain lacks the receptor sites for Mu.

† J53 (RP4) was provided by J. E. Beringer (John Innes Institute, Norwich), AB1157 and K12S by R. Devoret (C.N.R.S., Gif-sur-Yvette, France), and c600 rK⁻ mK⁻ by P. Kourilsky (Institut Pasteur, Paris). Phages Mu-1 and Mu-1 *cts* 62 were obtained from V. Enea (Rockefeller University, New York).

‡ Plasmid nomenclature as proposed by Novick *et al.* (1976).

E. coli, 75 µg ml⁻¹ for *P. solanacearum*, 100 µg ml⁻¹ for *R. meliloti*). In control experiments, donor and recipient cultures were filtered on to separate membranes and then incubated and plated under the same conditions as the crosses. Transfer frequencies are given per donor cell.

Characterization of P. solanacearum and R. meliloti transconjugants. *Pseudomonas solanacearum* transconjugants were tested for sensitivity to the temperate phage *a* by cross-streaking. Virulence was tested on tomato seedlings (variety Marmande) grown in test-tubes under sterile conditions. The test was carried out in a controlled environment cabinet at 30 °C, with a day-length of 16 h and 95 to 100 % humidity. Wilting of seedlings was observed 2 weeks after inoculation.

Rhizobium meliloti transconjugants were tested for unselected markers: sensitivity to the virulent phage II1, and inability to grow in anaerobic conditions in the presence of nitrate. Their symbiotic properties were checked on alfalfa seedlings (variety Milfeuil) grown aseptically in test-tubes: nodule formation and nitrogen fixation were measured 4 weeks after inoculation.

Lysogenization with Mu and titration of Mu. *Escherichia coli* strains were lysogenized as described by Taylor (1963). Mu lysates were prepared by the confluent lysis technique (Adams, 1959). Lysates of the thermoinducible mutant Mu *cts* were prepared by heat induction of a lysogenic liquid culture by growth at 43 °C for 1 h. Lysogeny was assayed by Mu production and immunity to the clear mutant Mu *c* 4. The presence of Mu *cts* prophage in *E. coli* was checked by testing for heat sensitivity at 43 °C. To determine the presence of Mu in a bacterial culture, 1 ml chloroform was added to 10 ml culture, and the mixture was shaken on a vortex mixer for 1 min, kept at room temperature for 15 min and then centrifuged for 10 min at 5000g. The supernatant was recentrifuged for 20 min at 5000g. The lysate was titrated (Adams, 1959) the same day and a sample was checked for sterility.

Chemicals. Kanamycin sulphate was a gift from Laboratoires Bristol (Paris, France); tetracycline hydrochloride was generously provided by Laboratoires Pfizer (Orsay, France). Streptomycin sulphate 'Rhodia' was obtained from Rhône-Poulenc, and rifampicin (Rif) from Schwarz Mann.

RESULTS

Insertion of Mu and Mu cts into the RP4 plasmid

Insertion of the wild-type Mu phage into RP4 was by lysogenization of *E. coli* 153 (RP4) after infection with a Mu lysate (Dénarié *et al.*, 1976). Integration of Mu into RP4 was detected by a method based on the dominance of the wild-type *c*⁺ allele over the *cts* mutation. The presence of a thermoinducible Mu *cts* prophage renders its *E. coli* host thermosensitive (Howe, 1973), whereas a strain which is diploid for the *c* gene and contains one Mu *cts* prophage and one Mu *c*⁺ prophage is not thermosensitive and grows at 42 °C (Howe, 1972). The lysogenized 153 (RP4) culture was crossed at 28 °C with *E. coli* AB1157 made thermosensitive by lysogenization with Mu *cts*. R⁺ transconjugants which became thermoresistant were assumed to have received an RP4::Mu plasmid. The insertion of the Mu prophage into RP4 was demonstrated by (i) the occurrence of zygotic induction in subsequent transfers to non-lysogenic Mu-resistant *E. coli* recipients (Schröder & Van de Putte, 1974), (ii) 100 % cotransfer of the *c*⁺ heat resistance phenotype with tetracycline and ampicillin (Amp) resistance, and (iii) the occurrence of polarized transfer of the chromosome when lysogenic R⁺ *E. coli* donors containing Mu on the chromosome were crossed with R⁻ lysogenic *E. coli* recipients (Dénarié *et al.*, 1976). No transfer could be detected when the R⁺ donors carried RP4 instead of RP4::Mu. For a given RP4::Mu, the direction of transfer depended

Table 2. Comparison of antibiotic marker transfer frequencies and of Mu phage production for two *E. coli* strains carrying respectively RP4 and RP4::Mu *cts*

Crosses were performed on filter membranes as described in Methods; *E. coli* × *R. meliloti* crosses were for 5 h, *E. coli* × *E. coli* crosses for 2 h.

R ⁻ recipient strains	Selected markers	<i>E. coli</i> R ⁺ donors	
		K12S (Mu <i>cts</i>) (RP4)	K12S (Mu <i>cts</i>) (RP4::Mu <i>cts</i>)
<i>R. meliloti</i> 2011 Str-r	Tet	9.2×10^{-3}	9.9×10^{-8}
<i>E. coli</i> AB1157 (Mu <i>cts</i>) Str-r	Amp and Tet	> 50 %	> 50 %
<i>E. coli</i> AB1157 Mu-r Str-r	Amp and Tet	54 %	1 %
No of infectious centres on an <i>E. coli</i> K12 indicator strain*		8.2×10^5	8.3×10^7
No. of thermosensitive clones for 20 R ⁺ AB1157 transconjugants†		0	20

* After crossing for 2 h the mixture was resuspended and treated with chloroform.

† Spot tests at 43 °C on modified L medium.

Table 3. Transfer frequencies of RP4, RP4::Mu and RP4::Mu *cts* in crosses between *E. coli* and *P. solanacearum*

R ⁺ donors	R ⁻ recipients	Transfer frequency (per initial donor)		
		RP4	RP4::Mu	RP4::Mu <i>cts</i>
<i>E. coli</i> AB1157 (Mu <i>cts</i>) Str-r	<i>P. solanacearum</i> GM11000	10^{-4}	2×10^{-4}	2×10^{-4}
<i>P. solanacearum</i> GM11000	<i>E. coli</i> AB1157 Str-r Mu-r	1.3×10^{-5}		3×10^{-7}
	<i>E. coli</i> AB1157 Str-r (Mu <i>cts</i>)	6×10^{-5}	7.8×10^{-7}	

on the orientation of the Mu prophage on the chromosome and the orientation of the Mu prophage in RP4 could be deduced from the direction of chromosome transfer as proposed by Zeldis, Bukhari & Zipser (1973).

Transfer of RP4::Mu plasmids from *E. coli* K12 to *R. meliloti* 2011 occurred at a much lower frequency than transfer of normal RP4. This observation led to a simple method for detecting the integration of the thermoinducible mutant Mu *cts* into RP4. An *E. coli* J53 (RP4) culture was lysogenized with Mu *cts* as described by Taylor (1963); such a culture should contain J53 (Mu *cts*) (RP4) and J53 (RP4::Mu *cts*) bacteria. Independent lysogenic clones were crossed with a streptomycin-resistant derivative of *R. meliloti* by spotting on selective C Str Tet agar plates; control spot crosses were made with an *E. coli* recipient lysogenic for Mu. The clone J53-29 was able to transfer its R factor to *E. coli* but not to *R. meliloti* and was therefore suspected of carrying an RP4::Mu *cts* plasmid. J53-29 and J53 (RP4) were crossed with a K12S (Mu *cts*) R⁻ recipient to obtain two isogenic strains differing only in their R factor: K12S (Mu *cts*) (RP4) and K12S (Mu *cts*) (RP4::Mu *cts*).

The results of the crosses of these two strains with different R⁻ recipients are presented in Table 2. The two R factors were transferred at the same frequency when the recipient *E. coli* strains were lysogenic for Mu and had the same modification-restriction system. When the recipient strain was a non-lysogenic Mu-resistant mutant of *E. coli*, the transfer frequency of RP4::Mu *cts* was decreased and the number of infectious centres was clearly increased: these two results are characteristic of zygotic induction. Clones of R⁺ transconjugants of this cross

Table 4. *Transfer frequencies of RP4, RP4::Mu and RP4::Mu cts in crosses between E. coli K12 and R. meliloti 2011 strains*

R ⁺ donors	R ⁻ recipients	Transfer frequency (per initial donor)		
		RP4	RP4::Mu	RP4::Mu <i>cts</i>
<i>E. coli</i> AB1157 (Mu <i>cts</i>) Str-r	<i>R. meliloti</i> 2011	3×10^{-4}		1.2×10^{-8}
<i>E. coli</i> J53 (Mu <i>cts</i>)	<i>R. meliloti</i> 2011 Str-r			
	Not heat treated	3×10^{-4}	2×10^{-9}	
	Heat treated*	3×10^{-4}	2×10^{-8}	
<i>E. coli</i> K12S (Mu <i>cts</i>)	<i>R. meliloti</i> 2011 Gly ⁻ Str-r	7×10^{-5}		1.9×10^{-8}
<i>R. meliloti</i> 2011 Str-r Gly ⁻	<i>R. meliloti</i> 2011 Str-r Rif-r	2.9×10^{-3}		3.5×10^{-3}
<i>R. meliloti</i> 2011 Str-r Gly ⁻	<i>E. coli</i> K12 Rif-r	1.7×10^{-2}		6×10^{-7}
	<i>E. coli</i> K12 (Mu <i>cts</i>) Rif-r	6.4×10^{-3}		8.3×10^{-8}
	<i>E. coli</i> C600 rK ⁻ mK ⁻ Rif-r	3.5×10^{-4}		2.8×10^{-6}
	<i>E. coli</i> C600 rK ⁻ mK ⁻ (Mu <i>cts</i>) Rif-r	6.4×10^{-2}		2.4×10^{-8}

* Six min at 47 °C.

were purified at 28 °C and tested at 43 °C for thermosensitivity: all the R⁺ strains which received the R factor from K12S (Mu *cts*) (RP4::Mu *cts*) were thermosensitive, whereas all the R⁺ strains which received RP4 from the K12S (Mu *cts*) (RP4) control were not. Therefore the modified RP4 detected in J53-29 carries a Mu *cts* prophage.

Introduction of Mu into P. solanacearum

As indicated in Table 3, the three plasmids, RP4, RP4::Mu and RP4::Mu *cts* were transferred to *P. solanacearum* GM11000 at about the same frequency, suggesting that the conjugative transfer of the Mu genome into *Pseudomonas* does not result in detectable zygotic induction or restriction. Twelve kanamycin-resistant transconjugants from each of the three matings were purified twice on minimal medium MM Kan and tested for the presence of an unselected RP4 marker (Tet-r); each clone was Tet-r. They were also sensitive to the *a* phage and formed white fluidal colonies on BGT agar plates. The Str-r unselected marker of the *E. coli* donors was not transferred. Therefore all the transconjugants tested were R⁺ derivatives of *P. solanacearum*. When one clone from each of three matings was tested for virulence on axenic tomato seedlings, typical wilting occurred. These three clones, GM11000 (RP4), GM11000 (RP4::Mu) and GM11000 (RP4::Mu *cts*), were used in subsequent experiments. In order to test whether the Mu prophage had been transferred with the plasmid, the plasmids were transferred back to *E. coli*. GM11000 (RP4) and GM11000 (RP4::Mu *cts*) were crossed with an *E. coli* AB1157 Str-r R⁻ recipient which was made resistant to Mu to avoid lysogenization by free Mu particles. R⁺ *E. coli* derivatives were purified twice on selective medium and tested for growth at 28 and 43 °C: R⁺ derivatives from the RP4::Mu *cts* cross were thermosensitive and produced phages whereas those from the RP4 cross were not. GM11000 (RP4) and GM11000 (RP4::Mu) were crossed with an *E. coli* Str-r recipient lysogenic for Mu *cts* and therefore thermosensitive. The purified *E. coli* transconjugants from the control (RP4) were still thermosensitive; but those from the RP4::Mu cross were able

to grow at 43 °C. These results indicate that the Mu *cts* prophage and the dominant *c*⁺ gene of the Mu prophage were present in the *P. solanacearum* donor.

The frequency of transfer of RP4::Mu and RP4::Mu *cts* within *P. solanacearum* GM1000 derivatives was not significantly different from that of RP4 indicating that if zygotic induction occurred it did not result in a detectable lethal effect or destruction of the vector plasmid.

Introduction of Mu into R. meliloti

The spontaneous mutation frequency to tetracycline resistance (10 µg ml⁻¹) for *R. meliloti* strain 2011 was less than 10⁻¹⁰. Therefore by using selective media containing tetracycline, it was possible to detect transfer of RP4 at a very low frequency. Derivatives of *E. coli* K12 carrying RP4, RP4::Mu and RP4::Mu *cts* were crossed with R⁻ *R. meliloti* recipients. Table 4 shows that antibiotic resistance was transferred at a much lower frequency when the R⁺ donors carried a Mu *c*⁺ or Mu *cts* prophage inserted in the RP4 plasmid.

The transfer frequency was increased approximately 10-fold when the recipient strain was heat treated before mating. The R⁺ transconjugants from the RP4 cross had a normal appearance on the selective agar plates, whereas the R⁺ transconjugants from RP4::Mu and RP4::Mu *cts* crosses formed small colonies. Eight R⁺ transconjugant colonies from each cross were purified three times on selective medium and shown to be *R. meliloti* as described in Methods. To check that there was no contamination by *E. coli*, deep soft agar tubes of L medium enriched with glucose and nitrate were inoculated with the R⁺ clones: no growth occurred in anaerobic conditions. The results indicated that the R⁺ transconjugants were pure cultures of *R. meliloti*. Given the very low frequency of transfer of the plasmids carrying Mu the question arose as to whether the Mu prophage was still present in the transconjugants. Eight R⁺ *Rhizobium* clones (from the RP4::Mu *cts* cross) were crossed with *E. coli* K12 strains. In only one cross were *E. coli* R⁺ progeny shown to be able to produce plaque-forming phage. Therefore, unlike *P. solanacearum* derivatives, only some of the R⁺ *Rhizobium* transconjugants seemed to carry RP4 with a non-defective prophage.

When crosses were performed within derivatives of *R. meliloti* 2011, the transfer frequencies of the different types of RP4 plasmids were high and similar, irrespective of whether they carried a non-defective prophage or no prophage. This indicates that no detectable zygotic induction occurs in *Rhizobium*, even if a non-defective prophage is transferred on RP4.

The 'small colony' phenotype was transferred with the antibiotic resistance characters within *R. meliloti* 2011 mutants and seemed, therefore, to be determined by the plasmids regardless of whether they carry a defective or a non-defective prophage. This suggests that R⁺ *Rhizobium* clones which were shown not to transfer RP4::Mu *cts* back to *E. coli* carried RP4 derivatives different from the original RP4, probably carrying a part of the Mu prophage. When spread on media that did not contain tetracycline, the 'small colony' clones segregated large colonies at a frequency of about 1 %: these large colonies were sensitive to tetracycline and presumably had lost their plasmids. The 'small colony' phenotype was not observed in *P. solanacearum*.

Production of Mu phage by P. solanacearum and R. meliloti

One clone of *P. solanacearum* and one of *R. meliloti* able to transfer RP4::Mu *cts* back to *E. coli* were studied. These new hosts produced infectious particles. Chloroform-treated supernatants of *P. solanacearum* and *R. meliloti* (RP4::Mu *cts*) or (RP4) cultures were assayed for plaque formation on different *E. coli* K12 strains. Both (RP4::Mu *cts*) cultures

Table 5. Titration on different *E. coli* strains for plaque-forming units present in the supernatant of cultures of *P. solanacearum* GM1000 and *R. meliloti* 2011 carrying an RP4::Mu *cts* plasmid

Pseudomonas solanacearum cultures were incubated at 30 °C for 8 h in aerated liquid medium.
Rhizobium meliloti cultures were grown at 35 °C for 6 h in aerated liquid medium.

Phage producing strains		Indicator strains				
		Wild-type strain not heat treated	K12S treated at 51 °C for 10 min	Restriction deficient mutant		Mu resistant mutant ABI157 Mu-r treated at 51 °C for 10 min
				Non-lysogenized c600	Lysogenized c600 (Mu)	
<i>P. solanacearum</i> GM1000 (RP4::Mu <i>cts</i>)	p.f.u. ml ⁻¹ Relative e.o.p.	80 2.5 × 10 ⁻⁴	1.5 × 10 ⁴ 5 × 10 ⁻²	3.0 × 10 ⁵ 1	< 1	< 1
<i>R. meliloti</i> 2011 Str-r Gly ⁻ (RP4::Mu <i>cts</i>)	p.f.u. ml ⁻¹ Relative e.o.p.	2 × 10 ² 0.4	Not tested	5 × 10 ² 1	< 1	< 1

produced phages able to plaque on a restriction and modification deficient (rK⁻ mK⁻) *E. coli* C600 indicator strain. *Pseudomonas solanacearum* and *R. meliloti* carrying RP4 did not produce phage. Cultures of Mu-sensitive *E. coli* K12 were used as a control in each experiment to rule out the possibility of aerial contamination of the cultures by Mu; all the controls were negative.

Since no plaques were formed on *E. coli* indicator strains lysogenic for or resistant to Mu, it appeared that the phage produced by *Pseudomonas* and *Rhizobium* was Mu. To confirm this, its mutagenic ability was tested. Plaques were inoculated into modified L broth, grown and plated on L agar. The resulting *E. coli* C600 lysogenic clones were immune to Mu *c* and thermosensitive. When *E. coli* clones lysogenic for the phage derived from *Pseudomonas* were replica-plated on minimal medium, approximately 1 % had acquired new auxotrophic mutations. These results provide strong evidence that the phage was Mu.

At 30 °C more vigorous aeration of the cultures increased phage production from about 5 × 10² to 3 × 10⁵ plaque-forming units (p.f.u.) ml⁻¹ in *P. solanacearum* and from 5 to 50 p.f.u. ml⁻¹ in *R. meliloti*. Under these conditions spontaneous phage production in *P. solanacearum* was of similar frequency to that in *E. coli* K12. The efficiency of plating (e.o.p.) of *P. solanacearum* lysates on the rK⁺ mK⁺ *E. coli* was much lower than on the rK⁻ mK⁻ C600 strain, demonstrating that the phage was modified in a different manner to that grown in *E. coli* K12 and was severely restricted by the K restriction system. On the other hand the Mu phages produced by *Rhizobium* were probably insensitive to the K restriction system (Table 5).

Production of Mu *cts* from *Pseudomonas* and *Rhizobium* was decreased when the cultures were shifted to 40 °C for 3 h (one generation time) and then transferred to 28 °C for 6 h, whereas in *E. coli* (Mu *cts*) this temperature shock would have induced phage production. This is further evidence that Mu phages were not produced by an *E. coli* donor contaminant.

DISCUSSION

We have shown that Mu is able to integrate into the R factor RP4 which has a guanine + cytosine (GC) content quite different from that of *E. coli* chromosomal DNA. This result suggests that differing GC contents should not prevent integration of Mu into DNA of

bacteria genetically distant from the Enterobacteriaceae. By inserting Mu into the broad host range plasmid RP4 the Mu genome has been introduced into new bacterial hosts quite distant phylogenetically from the original *E. coli* K12 host. The transfer frequencies of RP4::Mu, RP4::Mu *cts* and RP4 into *P. solanacearum* GMI1000 were similar and high and the Mu prophage was undamaged. This suggests that during conjugative transfer GMI1000 does not restrict Mu DNA modified by the *E. coli* K system and that significant zygotic induction does not occur. If it does occur, it does not lead to death of the recipient or destruction of the vector plasmid as it does for Mu integrated in F in *E. coli* (Schröder & Van de Putte, 1974; Razzaki & Bukhari, 1975).

The transfer of RP4::Mu from *E. coli* to *R. meliloti* 2011 exhibits three features: (i) the presence of Mu in RP4 lowers its transfer frequency by a factor of 10^4 to 10^5 ; (ii) only some of the R⁺ transconjugants carry a non-defective Mu prophage; and (iii) R⁺ transconjugants exhibit a small-colony phenotype on agar media. The decrease in transfer frequency could be due to strong zygotic induction which destroys the plasmid or kills the recipient due to expression of the killing functions of the phage, or to destruction of the plasmid by an efficient restriction mechanism present in the new host, or to a combination of these two mechanisms. Inactivation of restriction processes by high temperature was first demonstrated in *E. coli* (Uetake, Toyana & Higawara, 1964; Schell & Glover, 1966), and has also been shown in *R. leguminosarum* L4 (Schwinghamer, 1966). A similar heat sensitivity was recently shown for the restriction mechanism of *C. freundii* towards the bacteriophage Mu (de Graaf *et al.*, 1973). Heat treatment of the recipient rhizobia increased their ability to receive RP4::Mu about 10-fold, suggesting that restriction may be involved. The small-colony phenotype may be due to the continuous expression of some gene(s) of Mu or to the increase in size of the plasmid.

In the two new hosts, the Mu genome is expressed and plaque-forming phages are produced. The production of such phages is higher for *P. solanacearum* than for *R. meliloti*. Inability to make heat induced lysates could be associated with the inability of these two strains to grow at 40 °C. Differences in the steps involved in expression of the Mu genome in *E. coli*, *Pseudomonas* and *Rhizobium* have not yet been investigated. Isolation of Mu phage mutants able to multiply more efficiently in the new hosts should be of great interest both for basic understanding of Mu phage replication and for a better use of Mu in genetic handling of *Rhizobium* and *Pseudomonas*. Extensive integration of Mu DNA into the *E. coli* genome occurs during the vegetative growth of the phage (Razzaki & Bukhari, 1975) and may be required for DNA replication (Schröder, Bade & Delius, 1974; Waggomer, Gonzales & Taylor, 1974). If we assume that the same mechanisms are operating in the new hosts it would follow that Mu is able to insert into the chromosome of *Pseudomonas* and *Rhizobium*. The fact that Mu can integrate into RP4 DNA which has a GC content of 58 % (Falkow *et al.*, 1974) is compatible with this hypothesis. The introduction of thermoinducible mutants of Mu into new hosts able to grow at 40 °C and having a chromosomal DNA density clearly different from that of *E. coli* and Mu, should simplify the molecular studies of excision and replication of the phage. More generally the RP4 plasmid could be used as a vector to introduce temperate bacteriophages into other resistant bacteria that are hosts for RP4.

RP4 can promote chromosomal gene transfer in *E. coli*, *P. solanacearum* and *Rhizobium* at low frequency (about 10^{-7} to 10^{-9}). We have recently shown that introduction of RP4::Mu into an *E. coli* strain carrying a Mu prophage in its chromosome promotes an oriented transfer of the chromosome and that RP4 prime factors can be isolated in *E. coli* by crossing lysogenic Mu *cts* (RP4::Mu) donors with *recA* recipient strains that are Mu lysogens (Dénarié *et al.*, 1976). Practically nothing is known about the genetics of Gram-negative

bacteria of interest for basic medical, agricultural or industrial purposes (Levinthal, 1974). It would therefore be useful to utilize RP4::Mu to develop their genetics.

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