Generalized Transduction in *Rhizobium leguminosarum*

By VICKY BUCHANAN-WOLLASTON

John Innes Institute, Colney Lane, Norwich NR4 7UH

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Generalized transduction in *Rhizobium leguminosarum* by two virulent bacteriophages, RL38 and RL39, is described. The transduction frequency was increased by ultraviolet irradiation of phage lysates. Cotransduction of auxotrophic and antibiotic resistance markers was shown, as was transduction of two P1 group R plasmids. Transduction between strains of *Rhizobium trifolii* and interspecific transduction from *R. leguminosarum* to *R. trifolii* occurred but no transduction from *R. trifolii* to *R. leguminosarum* was detected.

INTRODUCTION

Transduction of *Rhizobium* species has previously been reported only in *Rhizobium meliloti*. Kowalski (1967, 1970) showed that a number of *R. meliloti* phages were capable of generalized transduction; one (L5) transduced several auxotrophic markers and cotransduced a *leu* marker with symbiotic ineffectiveness (Kowalski, 1974). Sváb *et al.* (1978) showed that the phage 16.3 of *R. meliloti* is a specialized transducing phage for a cysteine marker.

Linkage maps, constructed by R plasmid-mediated recombination, are available for *R. meliloti* (Meade & Signer, 1977; Kondorosi *et al.*, 1977) and *R. leguminosarum* (Beringer *et al.*, 1978*a*). Interspecific recombination between *R. leguminosarum*, *R. trifolii* and *R. phaseoli* has also been shown (Johnston & Beringer, 1977). No transduction system for fine structure mapping has previously been reported for these other species. This paper describes generalized transducing phages, RL38 and RL39, which have been used for transduction in *R. leguminosarum* and *R. trifolii* and for transduction between these two species.

METHODS

Bacterial strains. These are shown in Table 1.

Media. Minimal medium (SY) was as described by Beringer (1974). Phages were propagated on modified SY medium containing 0.05 % (w/v) glucose instead of sodium succinate. Soft Y, used for top layers, was SY medium containing 0.6 % (w/v) Difco agar and was supplemented with sodium succinate, sodium glutamate and vitamin solution (as SY) before autoclaving. VS was Vincent's (1970) phage broth. *Escherichia coli* strains were grown on Oxoid nutrient agar (NA).

Antibiotics. These were used at the following final concentrations (mg l⁻¹): kanamycin sulphate, 20; streptomycin sulphate, 200; rifampicin, 20; tetracycline hydrochloride, 10; spectinomycin sulphate, 200; gentamicin sulphate, 10. For *E. coli*, gentamicin sulphate was used at 5 mg l⁻¹.

Phage isolation. Soil samples were collected locally and shaken overnight in an exponential phase culture (approx. 50 ml) of *R. leguminosarum* strain 1682. The culture was then centrifuged and the supernatant was filtered through a 0.45 µm pore size Millipore membrane. The filtrate was plated in soft agar top layers seeded with bacteria to detect phages. Plaques were picked and purified by two single-plaque isolations. Phages were stored in VS over chloroform.

Preparation of phage lysates and bacterial cultures. Phage lysates were prepared by the method described by Adams (1959). Bacteria and phages were mixed in a top layer to give confluent lysis after 2 d growth at 28 °C. VS (3 ml) was then added to the plates, left for 2 h to elute the phages and collected. Lysates were sterilized with chloroform. Titres of up to 10⁸ plaque-forming units (p.f.u.) ml⁻¹ were obtained by this method.

Table 1. *Bacterial strains and plasmids*

Strain	Species	Genotype	Reference or source
1055	<i>Rhizobium leguminosarum</i>	<i>ura-14 met-14</i>	This laboratory
1682	<i>R. leguminosarum</i>	<i>phe-1 trp-14 rif-66 str-273 spc-15</i>	This laboratory
1497	<i>R. leguminosarum</i>	<i>phe-1 trp-14 rif-66 spc-15</i>	This laboratory
1086	<i>R. leguminosarum</i>	<i>liv-1 cys-9 str-91</i>	This laboratory
1683	<i>R. leguminosarum</i>	<i>phe-1 trp-14 str-36 spc-15</i> (R68.45)	This laboratory
1057	<i>R. leguminosarum</i>	<i>ura-14 met-14 rif-74</i>	This laboratory
1245	<i>R. leguminosarum</i>	<i>ser-1</i> (R68.45)	This laboratory
1380	<i>R. leguminosarum</i>	<i>phe-1 met-12 rib-2 str-69</i>	This laboratory
1446	<i>R. leguminosarum</i>	<i>trp-14 ura-14 str-69</i>	This laboratory
1504	<i>R. leguminosarum</i>	<i>phe-1 ade-71 str-112</i>	This laboratory
1569	<i>R. leguminosarum</i>	<i>phe-1 ade-72 rif-304</i>	This laboratory
1703	<i>R. leguminosarum</i>	<i>trp-49 his-16 rif-19 spc-52</i>	This laboratory
6710	<i>R. trifolii</i>	<i>rif-258</i>	Johnston <i>et al.</i> (1978 <i>b</i>)
T72	<i>R. trifolii</i>	<i>str-144 liv*-9</i>	This laboratory
T153	<i>R. trifolii</i>	<i>str-144 spc-26 trp*-59</i>	This laboratory
T94	<i>R. leguminosarum</i>	<i>ura-14 trp-16 str-69 ade*-91</i>	Beringer <i>et al.</i> (1978 <i>b</i>)
2202	<i>R. leguminosarum</i>	<i>phe-1 trp-12 str-37</i> (pPH1JI)	Hirsch (1978)
402	<i>Escherichia coli</i>	<i>pro met nal</i>	E. Meynell
	Plasmid	Antibiotic resistances†	Reference
	R68.45	Ap Km Tc	Haas & Holloway (1976)
	pPH1JI	Ge Sp	Hirsch (1978)

All *R. leguminosarum* strains are derivatives of wild-type strain 300 (Johnston & Beringer, 1975). All *R. trifolii* strains are derivatives of wild-type strain 6621 (strain 204 in the Rothamsted Culture Collection). Auxotrophic markers were introduced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis or transposon (*) mutagenesis; antibiotic resistances were spontaneous.

* T strains carry the transposon Tn5 and insertion has induced the auxotrophy indicated by the asterisk.

† Abbreviations for plasmid-mediated antibiotic resistances: Ap, ampicillin; Km, Kanamycin; Tc, tetracycline; Ge, gentamicin; Sp, spectinomycin.

Recipient bacteria for transduction experiments were prepared by growing shaken cultures at 28 °C in VS to late-exponential phase. Bacteria were centrifuged, washed twice in 0.9 % (w/v) saline and resuspended in VS.

Transduction. Equal volumes of recipient bacteria and phage lysate were mixed to give a multiplicity of infection of about one. The transduction mixture was incubated at 28 °C for 3 h. To select for transduction of prototrophic alleles or R plasmids, the mixture was then plated directly on selective media. To select for transduction of rifampicin and/or streptomycin resistances, the mixture was plated on minimal medium without antibiotic, left for 18 h at room temperature to allow for phenotypic expression, and then overlaid with soft Y (3 ml per plate) containing rifampicin and/or streptomycin. Plates were incubated for 6 d at 28 °C before scoring.

Ultraviolet light (u.v.) treatment of phages. Phage lysate (3 ml) was placed in a plastic Petri dish (50 mm diam.) and irradiated with ultraviolet light at a dose rate of approximately 2 J m⁻² s⁻¹.

RESULTS

Isolation of transducing phages

Rifampicin and streptomycin resistance markers have been shown by conjugation to be closely linked on the *R. leguminosarum* chromosome (Beringer & Hopwood, 1976). Therefore cotransduction of these two markers should be possible and selection for coinheritance of both allows rapid screening for transducing phages since the possibility of selecting spontaneous mutants is negligible. Twenty-six different phage isolates were tested for their ability to transduce these markers from *R. leguminosarum* strain 1682 to strain 1055. Two of these, RL38 and RL39, transduced at frequencies greater than 10⁻⁸. Phage RL38 transduced at a frequency tenfold higher than RL39 and was used in the majority of experiments.

Fig. 1. Effect of u.v. irradiation on the viability of RL38 (■) and RL39 (□) and on the frequency of cotransduction of rifampicin and streptomycin resistance by RL38 (●) and RL39 (○) from *R. leguminosarum* strain 1682 to 1055.

Effect of u.v. irradiation

Ely & Johnson (1977) showed that transduction in *Caulobacter crescentus* by phage ϕ Cr30 was improved when phage lysates were exposed to u.v. irradiation to 0.1 % survival. A similar effect was found for RL38 and RL39 (Fig. 1). The optimum time of u.v. irradiation was 5 min and this treatment was used routinely in all subsequent experiments. During this period of irradiation the plaque-forming titre of the lysate fell from approximately 10^9 to approximately 10^5 p.f.u. ml⁻¹ (Fig. 1).

Phage properties

RL38 and RL39 are probably virulent phages. All the phages isolated to date produced turbid plaques on the medium used for propagation of *R. leguminosarum* phages. However, the plaques of RL38 and RL39 were clearer than those of known lysogenic *R. leguminosarum* phages RL27 and RL30 (Buchanan-Wollaston, 1977). Bacteria from the centres of several turbid plaques were streaked to give single colonies and tested for sensitivity to the relevant phage by spot tests. All of 128 single colonies tested were sensitive, indicating that the turbidity of the plaques was not due to the formation of lysogenic bacteria.

Phages RL38 and RL39 adsorb very slowly to the bacteria; approximately 90 % adsorption occurred in 3 h (unpublished results). Washing the bacteria with 0.9 % (w/v) saline improved adsorption, presumably by removing some of the surface polysaccharide.

Linkage of *rif*, *str* and *spc* alleles

In *R. leguminosarum*, *rif*, *str* and some *spc* alleles map very closely by R68.45-mediated recombination (unpublished results). Cotransduction of the three markers by RL38 was shown (Table 2), indicating that they are closely linked. Transductants carrying the donor genotype always formed the largest class, and therefore the distances between the three markers must be small compared with the length of the transduced fragments.

The sequence *rif-str-spc* is consistent with the data in Table 2. In the three crosses the smallest transductant class was produced either by a quadruple crossover event (first and

Table 2. *Three-factor transduction crosses to determine the order of rif, str and spc markers in R. leguminosarum*

Donor strain	Recipient strain	Selected allele	Class of transductants*	No. in class†	Crossovers required in intervals:	
1497	1086	<i>rif</i>	<i>rif</i> ‡ <i>rif str</i> <i>rif spc</i> <i>rif str spc</i>	90 (21 %) 94 (22 %) 210 (50 %) 28 (7 %)	1, 3 1, 2 1, 4 1, 2, 3, 4	
1683	1057	<i>str</i>	<i>str</i> ‡ <i>str rif</i> <i>str spc</i> <i>str rif spc</i>	18 (14 %) 5 (4 %) 64 (50 %) 41 (32 %)	1, 3 2, 3 1, 4 2, 4	
1682	1055	<i>rif</i>	‡ <i>rif</i> <i>rif str</i> <i>rif spc</i> <i>rif str spc</i>	75 (18 %) 58 (13 %) 8 (2 %) 283 (67 %)	1, 2 1, 3 1, 2, 3, 4 1, 4	

* See Table 1 for allele markers.

† Numbers in parentheses show the percentage of total recombinants.

‡ These classes could have arisen by a single mutation in the recipient and the numbers shown have been corrected for this. The proportion of spontaneous mutants on the control plates to total colonies on the transduction plates was calculated and this proportion of the number of colonies tested was subtracted from the number found to be of the relevant genotype.

third crosses), or by a double crossover occurring in the intervals between the three markers (second cross).

Transduction of auxotrophic markers

Table 3 shows the auxotrophic markers that were transduced to prototrophy in derivatives of *R. leguminosarum* strain 300. The frequencies shown are for RL38 and they varied only by a factor of 10 for different markers. Table 4 shows the cotransduction of auxotrophic markers by RL38. There is a correlation between the coinheritance frequencies obtained by transduction and those obtained by conjugation.

Transduction of plasmids

Two P1 group R plasmids, R68.45 and pPH1JI, were transduced by RL38 and RL39 (Table 5). These plasmids have molecular weights of 37.4×10^6 and 32×10^6 , respectively (Jacob *et al.*, 1977; Hirsch, 1978). Transductants were tested for the unselected drug resistance carried on the R plasmid and their ability to conjugate was tested in patch crosses (Johnston *et al.*, 1978*a*). Transductants were mixed with another *R. leguminosarum* strain, or with *E. coli* strain 402, left overnight and then replicated to selective media to check for R plasmid transfer. *Escherichia coli* crosses were replicated to NA supplemented with the relevant antibiotic. The ampicillin resistance carried on R68.45 is expressed in *E. coli* though not in *R. leguminosarum* (Beringer, 1974). In all cases, transduced plasmids carried the same drug resistances as the parent and remained conjugative.

Table 3. Transduction of auxotrophic markers in *R. leguminosarum*

Donor strain	Recipient strain	Selected allele	No. of colonies with selected phenotype	Transduction frequency (per recipient)	Spontaneous reversion frequency (per recipient)
1086	1497	<i>trp-14</i> ⁺	1734	3×10^{-6}	$< 10^{-8}$
1086	1497	<i>phe-1</i> ⁺	600	8×10^{-7}	1×10^{-8}
1497	1086	<i>cys-9</i> ⁺	2560	5×10^{-6}	4×10^{-8}
1683	1245	<i>ser-1</i> ⁺	328	3×10^{-6}	$< 10^{-8}$
1380	1446	<i>ura-14</i> ⁺	200	2×10^{-6}	$< 10^{-8}$
1446	1380	<i>met-12</i> ⁺	1120	6×10^{-6}	$< 10^{-8}$
1380	1504	<i>ade-71</i> ⁺	212	8×10^{-7}	$< 10^{-8}$
1446	1569	<i>ade-72</i> ⁺	436	2×10^{-6}	$< 10^{-8}$
1504	1703	<i>his-16</i> ⁺	540	7×10^{-6}	1×10^{-8}
1504	1703	<i>trp-49</i> ⁺	1040	4×10^{-6}	$< 10^{-8}$

Table 4. Cotransduction of auxotrophic markers in *R. leguminosarum*

Selected allele	Coinherited marker	Percentage coinheritance by transduction	Percentage coinheritance by conjugation*
<i>trp-14</i> ⁺	<i>ade-72</i>	2	70
<i>ade-72</i> ⁺	<i>trp-14</i>	0.5	
<i>trp-14</i> ⁺	<i>ade-91</i>	1	
<i>ade-71</i> ⁺	<i>rib-2</i>	18	86
<i>met-12</i> ⁺	<i>ura-14</i>	5	82
<i>ura-14</i> ⁺	<i>met-12</i>	10	

* From Beringer *et al.* (1978*a*).Table 5. Transduction of *R* plasmids in *R. leguminosarum*

Donor strain	Recipient strain	R plasmid	Selected resistance	Unselected resistance	Transducing phage	No. of colonies with selected phenotype	No. of colonies non-selected tested for markers and conjugative ability*	Transduction frequency
1683	1057	R68.45	Tc	Km Ap	RL38	40	31	1×10^{-7}
			Tc	Km Ap	RL39	39	33	1×10^{-7}
2202	1057	pPH1JI	Ge	Sp	RL38	47	15	2×10^{-7}
			Ge	Sp	RL39	65	30	2×10^{-7}

* These transductants were crossed with *E. coli* strain 402 and then tested for all drug resistance markers. In all cases tested the transduced plasmids remained conjugative and retained all the drug resistance markers of the parent.

Interspecific transduction

Phage RL38 formed plaques on *R. trifolii* strain 6621 and high phage titres could be prepared on this strain and its derivatives. The efficiency of plating of the phage was different on *R. leguminosarum* and *R. trifolii* though this difference did not seem to be due to normal restriction. Phage grown on derivatives of *R. leguminosarum* strain 300 plaqued with a tenfold higher efficiency on this strain than on *R. trifolii* strain 6621, but phage grown on derivatives of 6621 also had an increased efficiency of plating (five- to tenfold) on strain 300 compared with 6621.

Table 6 shows the transductions that were attempted between *R. leguminosarum* strains, between *R. trifolii* strains and between the two species. Strains carrying Tn5-induced auxo-

Table 6. *Transduction of transposon-induced auxotrophy in R. leguminosarum and R. trifolii using phage RL38*

Donor strain	Recipient strain	Selected allele	Coinherited marker	Transduction frequency	No. of colonies tested†
<i>R. leguminosarum</i> 1057	<i>R. trifolii</i> T72	<i>liv-9</i> ⁺	<i>kan</i> ⁺	4×10^{-6}	224
<i>R. leguminosarum</i> 1057	<i>R. trifolii</i> T153	<i>trp-59</i> ⁺	<i>kan</i> ⁺	4×10^{-6}	266
<i>R. trifolii</i> 6710	<i>R. leguminosarum</i> T94	<i>ade-91</i> ⁺	—	$< 10^{-8}$	—
<i>R. trifolii</i> 6710	<i>R. trifolii</i> T72	<i>liv-9</i> ⁺	<i>kan</i> ⁺	3×10^{-6}	180
<i>R. trifolii</i> 6710	<i>R. trifolii</i> T153	<i>trp-59</i> ⁺	<i>kan</i> ⁺	7×10^{-6}	82
<i>R. leguminosarum</i> T94	<i>R. trifolii</i> 6710	* <i>kan</i>	<i>ade-91</i>	8×10^{-6}	532
<i>R. trifolii</i> T72	<i>R. leguminosarum</i> 1057	* <i>kan</i>	—	$< 10^{-8}$	—
<i>R. trifolii</i> T72	<i>R. trifolii</i> 6710	* <i>kan</i>	<i>liv-9</i>	5×10^{-6}	425
<i>R. trifolii</i> T153	<i>R. leguminosarum</i> 1057	* <i>kan</i>	—	$< 10^{-8}$	—
<i>R. trifolii</i> T153	<i>R. trifolii</i> 6710	* <i>kan</i>	<i>trp-59</i>	3×10^{-6}	390
<i>R. leguminosarum</i> 1057	<i>R. leguminosarum</i> T94	<i>ade-91</i> ⁺	<i>kan</i> ⁺	2×10^{-5}	100
<i>R. leguminosarum</i> T94	<i>R. leguminosarum</i> 1057	* <i>kan</i>	<i>ade-91</i>	1×10^{-5}	100

**kan*, Selection for kanamycin resistance carried on Tn5.

† In all cases, transductants selected for one marker showed coinheritance of the unselected marker.

trophic markers (Beringer *et al.*, 1978*b*) were used in these transductions since they had two cotransducible markers – auxotrophy and kanamycin resistance. Transduction occurred at high frequency between *R. leguminosarum* strains and also at fairly high frequency between *R. trifolii* strains. Markers could be transduced from *R. leguminosarum* to *R. trifolii* but no transduction was detected in the reverse direction (frequency less than 10^{-8}).

DISCUSSION

RL38 and RL39 are the first phages reported to be capable of mediating generalized transduction in *R. leguminosarum*. Other transduction systems in *Rhizobium* species have utilized temperate phages, whereas RL38 and RL39 are almost certainly virulent. The use of such phages in transduction has been reported previously. In all cases, reduction in the killing potential of the phages was required, either by u.v. pretreatment (Ely & Johnson, 1977) or by the use of suppressible phage mutants (Drexler, 1970; Yasbin & Young, 1974) or temperature-sensitive mutations (Campos *et al.*, 1978). U.v. treatment of virulent phages increases the frequency of transduction because it reduces the number of plaque-forming particles and hence the killing potential of the phage lysates (Ely & Johnson, 1977).

U.v. treatment has previously been shown to increase the transducing frequency of several temperate phages, notably P22 (Garen & Zinder, 1955), λ (Arber, 1958) and P1 (Luria *et al.*, 1960). Benzinger & Hartman (1962) found that, in P22, this increase appeared to be due to increased recombination, resulting in a higher ratio of complete to abortive transductants. The decrease in transduction frequency caused by excess u.v. irradiation of the lysates may be due to inactivation of the bacterial DNA carried in the transducing phage particles, and/or to destruction of phage functions necessary for adsorption and injection of DNA into the bacterial cell.

Transduction by RL38 and RL39 is generalized since markers from all around the chromosome were transduced. The only detectable difference between the two phages was in their transducing frequency. Their plaque morphology was the same and their cotransduction frequencies of linked markers were very similar; this may indicate a similarity in size.

The use of transduction by RL38 and RL39 to show complete coinheritance of Tn5-induced auxotrophy with kanamycin resistance, as well as a complete correlation of loss of kanamycin resistance with transduction of the Tn5-induced auxotrophy to prototrophy, has already been reported for *R. leguminosarum* (Beringer *et al.*, 1978*b*). This paper shows

the same result for *R. trifolii* and demonstrates that transduction can occur from *R. leguminosarum* to *R. trifolii* although not, apparently, in the reverse direction.

Phage RL38 had a higher plaquing efficiency on *R. leguminosarum* than on *R. trifolii* regardless of which species was its previous host. It may be that adsorption to *R. trifolii* is slower so that fewer phage particles form plaques. The observation that transduction can occur from *R. leguminosarum* to *R. trifolii* but not from *R. trifolii* to *R. leguminosarum* cannot be explained by poor adsorption since phages grown on *R. trifolii* had a higher efficiency of plating on *R. leguminosarum*. A general system of restriction is also an unlikely explanation because DNA can be transferred from *R. trifolii* to *R. leguminosarum* by conjugation and stable recombinants are formed at similar frequencies to those obtained in crosses between strains of *R. leguminosarum* (Johnston & Beringer, 1977). Since transduction occurs in one direction, recombination difficulties due to lack of homology in the short section of DNA are unlikely to be the explanation.

The system of generalized transduction for *R. leguminosarum* and *R. trifolii* reported here will be useful for high-resolution mapping in these strains. The availability of interspecific transduction may allow fine structure mapping of host range genes.

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