Transfer of Antibiotic Resistance Plasmid RP1 into *Pseudomonas glycinea* and *Pseudomonas phaseolicola* *in vitro* and *in planta*

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SUMMARY

The wide host-range antibiotic resistance plasmid RP1 was transferred from *Pseudomonas aeruginosa* via *Escherichia coli* into *Pseudomonas glycinea*. The plasmid was then acquired by *Pseudomonas phaseolicola* both *in vitro* and *in planta* in *Phaseolus limensis* leaves and pods. This was the first step in the design of a model system to determine the possible epidemiological significance of antibiotic resistance plasmids in the control of plant disease.

INTRODUCTION

The possibility of R factor transfer among bacteria causing plant diseases which are routinely controlled by antibiotic therapy could have great epidemiological and economic implications. Recently, several studies have reported that phytopathogenic or phytosymbiotic species of bacteria were able to acquire plasmids *in vitro*. Chatterjee & Starr (1972*a,b*) have transferred the fertility plasmid F'*lac*⁺ and the resistance plasmid R100rd into *Erwinia* spp. The same workers have achieved chromosomal transfer (Chatterjee & Starr, 1973). Various workers have transferred group P plasmids (Holloway & Richmond, 1973) from *Pseudomonas aeruginosa* into *Rhizobium* spp. (Mattes *et al.* 1973; Dunican & Tierney, 1973; O'Gara & Dunican, 1973; Datta *et al.* 1971; Datta & Hedges, 1972). Datta & Hedges (1972) also transferred F'*lac*⁺ into *Rhizobium lupini*. Group P plasmids have been transferred to the phytopathogenic pseudomonads *P. glycinea*, *P. lachrymans*, *P. phaseolicola* and *P. syringae* (Panopoulos, Guimaraes & Schroth, 1973; Guimaraes, Panopoulos & Schroth, 1973, 1974) as well as the causative agent of crown gall, *Agrobacterium tumefaciens* (Datta & Hedges, 1972).

If plasmids have a role in the epidemiology of antibiotic resistance in plant disease, then they must be transmissible on or in plant tissue. No studies have been directed at this premise.

However, plasmids are transferable under natural or simulated natural conditions. *In vivo* transfer of plasmids has been reported in the intestinal tract of gnotobiotic or antibiotic treated mice, pigs and humans (Schneider, Formal & Baron, 1961; Jones & Curtis, 1969; Mitsuhashi, 1971). Weinberg & Statzky (1972) have evidence for plasmid transfer in sterile soil. These studies suggest that plasmids may be capable of movement from bacterium to bacterium in diverse natural environments.

This study was undertaken to determine if conjugal transfer of resistance plasmids was possible among species of phytopathogenic pseudomonads *in planta* as well as *in vitro*. This report forms the basis for a model upon which the epidemiological significance of *in planta* plasmid transfer in diseased plants may be tested.

Table 1. *Origins of bacterial isolates*

Bacterium	Isolate	Genotype	Origin†
<i>Escherichia coli</i>	x705	<i>arg leu Sm^rCb^slac⁺F⁻</i>	a
<i>Pseudomonas</i> spp.			
<i>P. aeruginosa</i>	PAT904 rev 1	Prototroph* <i>Cm^sSm^rRP1⁺</i>	b
<i>P. fluorescens</i>	PF015:4	<i>met Cm^sSm^rRP1⁺</i>	b
<i>P. glycinea</i> R-1	L113†	Prototroph <i>Sm^sCb^s</i>	c
<i>P. glycinea</i> R-2	L114	<i>cys thr Sm^sCb^s</i>	c
<i>P. glycinea</i> R-5	L115	Prototroph <i>Sm^sCb^s</i>	c
<i>P. glycinea</i> R-6	PG9	<i>met Sm^sCb^s</i>	d
<i>P. phaseolicola</i>	HB36	Prototroph <i>Sm^sCm^sCb^s</i>	d
<i>P. phaseolicola</i>	HB10Y	Prototroph <i>Sm^sCb^s</i>	e
<i>P. phaseolicola</i>	L124	<i>thi Sm^sCb^s</i>	f

* The criterion for prototrophy was growth on Davis' MA or MB.

† The L-designations refer to the author's strain code.

‡ (a) Roy Curtiss III, University of Alabama, Birmingham, U.S.A.; (b) R. H. Olsen, University of Michigan Medical School, Ann Arbor, U.S.A.; (c) B. W. Kennedy, University of Minnesota, St Paul, U.S.A.; (d) M. N. Schroth, University of California, Berkeley, U.S.A.; (e) A. K. Vidaver, University of Nebraska, Lincoln, U.S.A.; (f) u.v. mutant of HB10Y.

METHODS

Origin of RP1. The wide host-range group P plasmid RP1, which confers resistance to carbenicillin (Cb), tetracycline (Tc), and kanamycin (Km)/neomycin (Nm) (Lowbury *et al.* 1969; Olsen & Shipley, 1973), was received in *P. aeruginosa* PAT904 rev 1 from R. H. Olsen of the University of Michigan Medical School, Ann Arbor, U.S.A.

Origins of bacterial strains. These are given in Table 1.

Inoculum preparation. Cells were cultured in Davis' minimal broth medium (MB) or complete broth without glucose (CB) for *Escherichia coli* (Lederberg, 1950) at 25 °C and 140 rev./min on a shaker with a 2.5 cm rotational diameter for 18 to 22 h.

In vitro RP1 transfer. RP1 was transferred in CB with the final suspensions of donor and recipient cells each adjusted to 10⁶ to 10⁷ colony forming units (c.f.u.)/ml on the basis of E₆₀₀. The broth cultures were incubated under static conditions at 25 °C. Dilutions were streaked or spread on to the appropriate selective media to detect RP1⁺ recipients.

In planta RP1 transfer. Immature *Phaseolus limensis* (Fordhook 242 lima beans from W. Atlee Burpee Co., Ames, Iowa, U.S.A.) bean pods were inoculated by injection of 0.1 ml distilled-water suspensions of washed recipient and donor cells adjusted to 10⁶ to 10⁷ c.f.u./ml. Leaflets of trifoliolate leaves were inoculated by water congestion of the abaxial surface with a hand sprayer. Recipient and donor cells were applied consecutively. Leaflets were also lightly misted with bacterial suspensions to determine if transfer could take place on the leaf surfaces. The detached pods and leaflets were incubated at 25 °C under humid conditions (100 % relative humidity) until assayed.

Extraction of bacterial cells from plant material. A minimum of ten bean leaflets and five bean pods were surface-disinfected in 1.0 to 1.2 % (w/v) aqueous sodium hypochlorite for 1 and 5 min, respectively. The plant material was minced and blended to a paste. The paste was filtered through four layers of cheesecloth and rinsed with sterile water to a final volume of 100 ml. The filtrate was centrifuged, and the pellet washed twice by centrifugation at approximately 380 g for 10 min and resuspended in 100 ml of water. Ten misted leaves were produced as described in the previous paragraph and rinsed with sterile water to a final volume of 100 ml. Appropriate dilutions were plated on to selective media.

Selective media. Davis' minimal agar medium (Lederberg, 1950) was used unsupplemented

as minimal agar (MA), supplemented with 500 µg Cb/ml (Geopen provided by R. G. Meny and G. H. Stanley of Pfizer Inc., New York, U.S.A.) as MA + Cb, supplemented with 20 µg methionine (met) plus 25 µg streptomycin (Sm)/ml as MA + met + Sm, or supplemented with 20 µg Tc or Nm/ml as MA + Tc or MA + Nm. Eosin-methylene blue agar was fortified with 500 µg Cb/ml and used as EMB + Cb. After inoculation all selective media plates were incubated at 25 or 37 °C for 48 to 72 h.

Confirmation of RP1 transfer. Presumptive RP1⁺ recipients were screened for appearance of multiple antibiotic resistance by replica-plating (Lederberg & Lederberg, 1952) on MA plus the appropriate antibiotic(s) or by plating on Davis' complete agar medium (CA), (Lederberg, 1950) and examining the zones of inhibition (z.o.i.) around antibiotic-sensitivity discs.

Phage PRR1 specificity for plasmid-transformed cells. The male-specific phage PRR1 (Olsen & Shipley, 1973; Olsen & Thomas, 1974), specific for cells containing group P plasmids, was also supplied by R. H. Olsen. The phage was diluted in CB and allowed to adsorb to *P. glycinea* PG9 and PG9 RP1⁺ for 5 to 10 min. Dilutions were mixed with molten overlay agar (CB with 0.7 % agar) at 45 °C and poured on CA plates. Incubation was at 25 °C and any plaques were counted at 18 to 24 h after inoculation.

Host range determination. The host range of *P. glycinea* PG9 was compared with its plasmid-containing progeny to determine if any change had occurred due to the presence of RP1. The *Glycine max* (soybean) cultivars, Acme, Flambeau, Harosoy and Kanrich, the lima bean cultivar Fordhook 242, and the *Phaseolus vulgaris* (field beans) cultivar Romano pole beans, were included in the comparison.

Young primary leaves or nearly expanded trifoliate leaflets were inoculated by water congestion with bacteria washed twice by centrifugation and diluted to 10⁷ to 10⁸ c.f.u./ml in sterile, distilled water. The plants were observed periodically for 7 to 10 days in the greenhouse for the development of symptoms.

Stability of the plasmid in the recipient host cells. The stability of the plasmid host cell complex was compared in *P. phaseolicola* strains H1310Y and HB36 and *P. glycinea* strain PG9. Cells were cultured in MB with antibiotics so that only resistant cells could develop. When the cell concentration reached 10⁷ c.f.u./ml the cells were transferred to CB and diluted to 10⁵ c.f.u./ml. Overnight cultures were washed by centrifugation, diluted, and spread on MA mixed with required nutrients. When colonies appeared, they were replicated on to MA + Tc + Nm. Colonies were scored for growth on MA and on MA + Tc + Nm. Any colonies not developing on the antibiotic-fortified agar were considered to have lost the plasmid spontaneously.

Curing of plasmids. The concentration of acridine orange (AO) causing inhibition of growth of PG9 RP1⁺ cells in an overnight shake culture of MB + met was measured by E_{600} . Colonies which developed on MA + met, from cells exposed to AO, were replicated to MA + met + Cb and scored for growth. Colonies which failed to replicate on the b-fortified medium were considered to have been cured of the plasmid.

Transfer frequency. The proportion of recipient cells acquiring the resistance plasmid was determined by the formula:

$$\text{Transfer frequency} = \frac{\text{RP1}^+ \text{ recipient colonies on MA + Cb}}{\text{recipient colonies on MA}}.$$

Table 2. *In vitro* and *in planta* transfer of RP1 from *P. glycinea* PG9 met Sm^r RP1⁺ to *P. phaseolicola* HB36 Sm

Experiment	Mating period (h)	Colony forming units/ml mating suspension on :			Transfer frequency†
		MA (recipients)	MA + met + Sm (donors)	MA + Cb (RP1 ⁺ recipients)	
<i>In vitro</i>					
1	2.0	9.0 × 10 ⁶	8.0 × 10 ⁶	5.2 × 10 ⁵	5.8 × 10 ⁻²
2	2.0	7.5 × 10 ⁶	ND	8.0 × 10 ⁴	1.1 × 10 ⁻²
3	2.0	7.5 × 10 ⁶	ND	7.0 × 10 ⁵	2.7 × 10 ⁻²
4*	2.5	1.0 × 10 ⁷	ND	4.9 × 10 ⁴	4.9 × 10 ⁻³
5	2.0	6.0 × 10 ⁴	4.3 × 10 ⁶	1.3 × 10 ³	2.2 × 10 ⁻²
<i>In planta</i> (<i>Phaseolus lunatus</i> – Fordhook 242 lima beans)					
Pods	6.5	1.2 × 10 ⁶	ND	6.6 × 10 ⁵	5.5 × 10 ⁻¹
Pods*	2.5	5.0 × 10 ⁶	3.8 × 10 ⁶	2.1 × 10 ⁴	4.2 × 10 ⁻³
Leaves					
(congested)	3.5	3.4 × 10 ⁴	3.7 × 10 ⁶	2.8 × 10 ³	8.2 × 10 ⁻²
Leaves					
(misted)	3.5	2.4 × 10 ⁵	1.2 × 10 ⁷	7.5 × 10 ⁴	3.1 × 10 ⁻¹

ND, not determined.

* Inoculum cultured in MB and MB + met + Sm rather than CB.

† For formula, see Methods.

RESULTS

Direct transfer of RP1 from *P. aeruginosa* PAT904 or *P. fluorescens* PFO15.4 to phytopathogenic pseudomonads could not be accomplished. In the first series of transfer experiments, the phytopathogenicity of *P. phaseolicola* was used as the criterion for selection of RP1⁺ recipients. Cultures were mixed in CB and enriched for Cb^r overnight in CB + Cb. No discrete lesions developed when the mixture of donor and recipients was inoculated on to Fordhook lima beans, although both PAT904 and PFO15.4 were re-isolated in nearly pure cultures. Selections of RP1⁺ recipients were also attempted on the basis of auxotrophy versus prototrophy and of antibiotic resistance versus susceptibility. Similarly, no RP1⁺ recipients were isolated when the donors PAT904 Cm^sRP1⁺ and PFO15.4 met Cm^s RP1⁺ were mated with *P. phaseolicola* L124 thi Cm^r Cb^s in CB and selection made in MB + Cb plus 200 µg Cm and 20 µg thiamine/ml.

Since direct transfer of RP1 from PAT904 and PFO15.4 was not detected, the plasmid was transferred into *E. coli*. This new host was used in subsequent experiments as a plasmid donor to phytopathogenic pseudomonads. Strain PAT904 was crossed with *E. coli* x705 in CB, and selection was made by streaking or spreading the mating suspensions on EMB + Cb. The distinct morphology of *E. coli* lactose-fermenting (Lac⁺) colonies on EMB allowed differentiation from the light coloured *Pseudomonas* colonies. The phenotype, x705 RP1⁺, was recovered from 5.6×10^{-3} of the recipient cells. The *E. coli* lac⁺ Cb^r colonies were found to have acquired resistance to Tc, Nm and Km when subcultured.

RP1 was readily transferred from *E. coli* x705 RP1⁺ *in vitro* and *in planta* (in pods) to *P. glycinea* PG9 and *P. phaseolicola* HB10Y. Selection for clones of the RP1⁺ phytopathogens was also made by streaking or spreading suspensions of mating cells on EMB + Cb. In this case the lac⁺ Cb^r *Pseudomonas* colonies were isolated from the streak plate. *In vitro* and *in planta* (in congested leaves) transfer frequencies of RP1 from x705 to PG9 were 2.8×10^{-3} and

Fig. 1. Representative data for the acquisition of RP1 during *in vitro* mating of *P. glycinea* PG9 met Sm^r RP1⁺ (donor) with *P. phaseolicola* HB36 Sm^s (recipient). The cells were plated on MA + Cb after agitation and dilution of the mating suspensions.

Table 3. Confirmation of RP1 transfer from *P. glycinea* PG9 met Sm^r RP1⁺ to *P. phaseolicola* HB36 Sm^s by demonstration of multiple antibiotic resistance in colonies replicated from MA + Cb

Experiment	Number of colonies			
	Replicated from MA + Cb	Growing on		
		MA + Tc	MA + Nm	MA + Sm
<i>In vitro</i> mating				
1	102	91	91	0
2	399	399	0	0
<i>In planta</i> mating (pods)				
3	355	353	354	0
4	205	199	203	1

1.6×10^{-2} respectively. Plasmid transfer was confirmed by determining the recipient nutritional pattern and the plasmid antibiotic resistance markers in the same clone.

Further evidence for plasmid transfer to *P. glycinea* PG9 was derived from experiments with the P-specific phage PRR1. Numerous plaques were detected on lawns of PG9 RP1⁺ but none on PG9.

RP1 was successfully transferred from PG9 RP1⁺ to HB36 *in vitro* and *in planta* (Table 2). *In vitro* acquisition of the plasmid by HB36 had reached a steady state in about 50 min (Fig. 1). In 2 h, an average of 3.0×10^{-2} recipient HB36 cells acquired carbenicillin resistance.

Table 4. *Host reaction comparison of P. glycinea PG9 (R-6) and P. glycinea PG9 RP1⁺ on soybeans, lima beans and pole beans*

	Host reaction			
	Previously reported		Experimental	
	Reaction	Reference‡	PG9	PG9 RP1 ⁺
<i>Glycine max</i>				
Acme*	R	a	I	I
Flambeau*	S	a	S	S
Harosoy*	R	a	R	R
Kanrich†	—	—	S	S
<i>Phaseolus lunatus</i>				
Fordhook 242†	S	b	S	S
<i>P. vulgaris</i>				
Romano†	—	—	S	S

R, resistant reaction; I, intermediate reaction; S, susceptible.

* Received from B. W. Kennedy, University of Minnesota, St Paul, U.S.A.

† Received from the W. Atlee Burpee Co., Ames, Iowa, U.S.A.

‡ (a) Cross *et al.* (1966); (b) Schroth *et al.* (1971).

Table 5. *AO curing of RP1 from P. glycinea PG9 recipients*

AO ($\mu\text{g/ml}$)	Colonies developing on		Percentage of colonies failing to develop on MA + met + Cb
	MA + met	MA + met + Cb	
	Expt 1		
None	131	130	0.76
15	67	63	5.97
	Expt 2		
None	586	583	0.51
15	525	519	1.14
20	662	651	1.66
30	303	280	7.59

RP1 transfer from PG9 RP1⁺ to HB36 was confirmed by demonstrating multiple antibiotic resistance in the recipients by growth of colonies replicated from MA + Cb on to MA containing Tc and Nm. The chromosomal marker, *Sm^r*, in HB36 remained stable when tested by replicating colonies on to MA + Sm (Table 3). Clones of the RP1⁺ recipients were compared with parental strains for changes in their antibiotic sensitivity spectra consistent with acquisition of RP1. In all cases resistance to antibiotic discs containing Tc, Nm and Km was observed.

RP1 was transferred from PG9 RP1⁺ to HB10Y and HB36 and could be transferred back to PG9. However, when PG9 pathogenic race 6 (Cross *et al.* 1966) was mated with *P. glycinea* race 1 or race 5, transfer of the plasmid was not detected among approximately 10^8 c.f.u./ml. This was unexpected, since the organisms are very closely related.

No host range or pathogenicity changes caused by alterations of the cell anatomy or physiology due to the presence of the plasmid were detected. Strains PG9 and PG9 RP1⁺ (compared on four cultivars of soybean, and one each of lima bean and pole bean) gave identical responses (Table 4) which were in general agreement with the published reactions (Cross *et al.* 1966; Schroth, Vitanza & Hildebrand, 1971). Flambeau and Harosoy soybeans gave the expected susceptible and resistant reactions, Fordhook 242 lima beans were susceptible as

described, but Acme soybeans gave an intermediate rather than resistant reaction with both PG9 and PG9 RP1⁺. Even though this reaction differed from the expected one, it was consistent for both PG9 and PG9 RP1⁺.

The stability of the plasmid-host cell complexes formed with the phytopathogenic pseudomonads was found to vary greatly. Strain PG9 formed a very stable complex with less than 1.0 % natural curing (Table 5), whereas HB10Y formed an unstable complex and it often showed 'double' z.o.i. around antibiotic discs. There was a marked decrease in the turbidity of the bacterial lawn coincident with the z.o.i. of HB10Y recipient cells without the plasmid. However, growth did occur in the z.o.i., which indicated some resistance to all the plasmid antibiotic markers. Strain HB36 consistently lost the plasmid upon subculture if selection by multiple antibiotics was not maintained.

AO at 25 to 30 µg/ml was found to inhibit the growth of 18 to 25 h cultures of PG9 RP1⁺ cells. Two experiments showed that only 0.51 to 0.76 % of the colonies replicated from MA + met to MA + met + Cb failed to develop when no AO was incorporated in the medium. However, as the concentration of AO increased, more colonies failed to replicate on the Cb-fortified medium (Table 5), indicating plasmid-curing.

DISCUSSION

Conjugal transfer of resistance plasmids was accomplished *in vitro* and *in planta* among the species of phytopathogenic pseudomonads investigated. The *in planta* transfer frequencies compared favourably with the *in vitro* ones. This, together with the ability of *E. coli* to transfer the plasmid into *Pseudomonas* spp. in bean pods, suggests that plasmid transfer between physiologically capable cells *in planta* is not limited by the host nor does it require a phytopathogen to phytopathogen relationship. Indeed, transfer appears to be independent of the host. The rapidity of the formation of mating pairs (see Fig. 1) and the mating of bacteria in water suspensions in bean pods and leaves, suggests that exogenous energy sources are unnecessary for the mating process. More important to the epidemiology of antibiotic resistance plasmids among phytopathogens is the rapidity of the exchange which occurs before the host plant's hypersensitive reaction could affect the physiology of recipient or donor cells. Bacterial populations do not decrease rapidly in many hypersensitive reactions (Allington & Chamberlain, 1949; Leben, Daft & Schmitthenner, 1968; Goodman, 1972; Keen & Kennedy, 1974). Therefore, the transfer of antibiotic resistance plasmids from pathogenic races of bacteria incompatible with the resistant host could also be completed.

The failure of *P. aeruginosa* and *P. fluorescens* to transfer RP1 to *P. phaseolicola* and *P. glycinea* PG9 directly, and the observation that *P. glycinea* PG9 could not transmit RP1 to *P. glycinea* races 1 and 5, may suggest interference of bacteriocins rather than exclusion by an incompatible host. Indeed, bacteriocins have been found among isolates of *P. phaseolicola* (8 %) and *P. glycinea* (55 %) and other phytopathogens (Vidaver *et al.* 1972). Another possible explanation for failure of plasmid transfer may involve the presence of incompatible plasmids in the recipient (Datta & Hedges, 1971; Willets, 1972).

The plant-pathological significance of the transfer of resistance plasmids in plants may be more than a simple model for the epidemiology of infectious resistance to antibiotic chemotherapy. It has been reported that RP1 and several related plasmids have fertility functions (Stanisich & Holloway, 1971; Holloway & Richmond, 1973). If these plasmids could mobilize and transfer chromosomes of phytopathogenic bacteria, insight could be gained into the nature of genes for 'pathogenicity' and 'virulence'. Genetic studies of pathogenicity in phytopathogenic pseudomonads may provide more profitable systems than

those employing intestinal pathogens of man, because of the intimate host-parasite relationships in the former, including specific host reactions and the existence of physiological races of pathogens.

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