Conjugative Transfer of Pseudomonas aeruginosa R Factors to Plant Pathogenic Pseudomonas spp.

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ABSTRACT

R18-1 and R91-1, two antibiotic resistance factors (R factors), specifying resistance to carbenicillin, and RP1, specifying resistance to carbenicillin, neomycin, kanamycin, and tetracycline, were transferred from *Pseudomonas aeruginosa* to *P. syringae*. RP1 was also transferred to *P. phaseolicola* and *P. glycinea*. R18-1 was transferred to these three species and to *P. pisi, P. marginalis, P. putida,* and *P. fluorescens*. The mechanism of transfer of R18-1, and presumably of the other R factors, was conjugation. The transfer frequency per donor cell was 10^{-6} to 2×10^{-3} in the case of R18-1, 3×10^{-7} to 2×10^{-5} for R91-1, and 5×10^{-4} to 2×10^{-2} for RP1. Acquisition of the R factors conferred upon the recipients capacity to produce penicillinase, ability to act as donors for antibiotic resistance, and (in the case of recipients of RP1) susceptibility to phage PRR1. Neither hypersensitive reaction on tobacco, nor pathogenicity of three species that were tested, was affected by inheritance of the R factors. Spontaneous segregation of R91-1 occurred at high frequency, and of R18-1 and RP1 at low or zero frequency, which in some cases was increased by sodium dodecyl sulfate, indicating a plasmid condition of the R factors in the plant pathogens. Acceptance of these and other P-group plasmids makes possible the search for a system for conjugative chromosome transfer in plant pathogenic *Pseudomonas* spp.

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Procaryotic fertility systems depend, in general, upon genetic elements, called sex factors, in the donor cells which may be either physically linked to, or separate from, the main bacterial chromosome (episomal and plasmid state, respectively) (15, 23). In their autonomous state such elements are transmissible independently of the chromosome to infertile recipient strains which, as a result, acquire donor ability. This property enables conjugative systems to be established in different bacteria, provided that they are within the host range of the sex factor.

A conjugative system in the plant pathogen *Erwinia amylovora* was established recently (5) following the successful transfer of F′ factors from other members of the Enterobacteriaceae to *Erwinia* spp. (4). This complemented work in progress in our laboratory, which was aimed at developing a conjugative system in the plant pathogenic pseudomonads for purposes of gene mapping and genetic analysis of pathogenicity. Since there was no information concerning natural fertility in these species and transferability of any known sex factors from other bacteria, we decided to investigate the transferability of sex factors from *Pseudomonas aeruginosa* to plant pathogenic pseudomonads.

Pseudomonas aeruginosa has several known sex plasmids (12, 13, 14, 15, 22, 23, 33). The plasmids chosen for study belong to a group of elements conferring resistance to antibiotics [antibiotic resistance (R) factors] and have been classified into a single plasmid compatibility group P-1 (2, 7). Group P-1 plasmids were first detected in hospital strains of *P. aeruginosa* and have been transferred to a wide variety of bacteria including soil saprophytes, various of the Enterobacteriaceae, Rhizobiaceae, the photosynthetic bacteria, and others (7, 8, 10, 25, 27, 34, 35). In addition, they promote chromosome transfer in *P. aeruginosa* (33) and *Escherichia coli* (J. E. Berringer, *personal communication*). The combination of wide host range and sex factor activity, prompted us to examine whether these R factors could be transferred to phytopathogenic pseudomonads. Abstracts of this work were published previously (27).

MATERIALS AND METHODS.—*Media.*—Mineral salts used were 1.0 g KH_2PO_4, 1.0 g Na_2HPO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.5 g sodium citrate, 1.0 g NaCl, and 4.0 g $(NH_4)_2SO_4$ per liter of water. One percent Proteose Peptone No. 3 was used as base for liquid complex medium. Glycerol was autoclaved separately and was added to both minimal and complex media at the 0.5 and 1% amounts with 2% agar. King's Medium B (KB) (18) was used as a solid complex medium. The Novick-Richmond medium (24) containing 0.3% casein hydrolysate and 0.3% yeast extract (0.3 CY) was used for detection of penicillinase-positive and -negative clones.

Antibiotics and DNAase.—Disodium carbenicillin was from Pfizer Inc., kanamycin sulfate from Bristol Laboratories, streptomycin sulfate and tetracycline hydrochloride from Nutritional Biochemicals Corporation, penicillin G and deoxyribonuclease II from Sigma Chemical Co., and neomycin sulfate from Pfizer, Inc.

DNAase was tested for activity on DNA-agar medium by placing a small grain of lyophilized enzyme on the agar for 20-30 minutes and observing clearing of the medium after flooding the petri dish with 1.0 N HCl.

Bacterial strains.—*Pseudomonas aeruginosa* 1822 (20) harbored the R-factor RP1, also named R1822 (14), which specified resistance to carbenicillin, neomycin, kanamycin, and tetracycline. A culture of the strain was obtained from B. W. Holloway and served as the original donor of multiple resistance to the four antibiotics in our experiments. The minimal inhibitory concentrations (MIC), determined by the serial dilution method (see below), were respectively $>$ 10,000 μg/ml, 500 μg/ml, 500

μg/ml, and 400 μg/ml. To facilitate screening of many potential recipients RP1 was introduced into the adenine-requiring *P. aeruginosa* JC9006. Tests showed that the R factor did not undergo genetic changes in this strain (Tables 2, 3, 4).

Pseudomonas aeruginosa PA08 (R18-1) and PA08 (R91-1) were products of crosses between the Lowbury strains 1822 and 9169, respectively, and strain PA08 (B. W. Holloway, *personal communication*). Both were highly resistant to carbenicillin (MIC > 10,000 μg/ml and 10,000 μg/ml, respectively), but in comparison with the Lowbury strains, 1822 and 9169, relatively sensitive to tetracycline (MIC > 200 μg/ml), kanamycin (MIC > 200 μg/ml), and neomycin [MIC > 50 μg/ml for PA08 (R18-1) and 12.5 μg/ml for PA08 (R91-1)]. Strain 9169, the source of the R91-1 factor, has similar resistance characteristics to strain 1822 but was not used in these experiments. PA08 (R18-1) and PA08 (R91-1) transfer only carbenicillin resistance (Table 3). Their original designations were PA08 (R18) and PA08 (R91), respectively, but were changed to the present ones for consistency with the recent nomenclature proposed by Holloway and Richmond (14).

According to these proposals, the transmissible elements specifying resistance to the four antibiotics in strain 1822 of Lowbury are designated RP1. Likewise, the elements specifying transmissible resistance to carbenicillin, which originated in the Lowbury strains 1822 and 9169, are designated R18-1 and R91-1, respectively. The source and identity of the recipient strains are given in Table 1.

Antibiotic resistance levels.—Quantitative tests for antibiotic resistance were performed by inoculating a series of test tubes containing geometric serial dilutions of individual antibiotics in complex liquid broth 1-2 ml/tube with approximately 10^2-10^3 cells/tube. The replica-plate technique was used when several clones were tested. Routine qualitative testing was done by streaking a loopful of bacterial suspension radially on KB and placing a 1-cm diameter Whatman No. 3 paper disk impregnated with a known amount of antibiotic in the center of the plate.

Mating procedure.—Unless specifically indicated otherwise, matings were conducted as follows: Fifteen- to 16-hour-old cultures of donor strains were grown in complex medium. Initially 500 to 1,000 μg/ml carbenicillin was added to prevent loss of antibiotic resistance during growth. This practice was later discontinued when it was proven unnecessary. The bacteria were subcultured in fresh broth to log phase, and were mixed with recipient cultures that had grown to the stationary phase in minimal glycerol medium. The growth temperature was 37 C for *P. aeruginosa* strains and 29 C for plant pathogenic isolates. The ratio of donor/recipient cells in the mating mixture varied from 1:1 to 1:100 but usually was 1:5 to 1:20. The volume of the mixture was 1-2 ml when mating was performed in test tubes, and 2-4 ml when mating was done in Erlenmeyer flasks. Incubation was at 29 C for 3-5 hours, without shaking unless otherwise noted. Serial dilutions were plated on media counterselective for the donor and recipient strains: minimal-glycerol + 500-1,000 μg/ml carbenicillin, in crosses with auxotrophic donors, KB + 500-1,000 μg/ml carbeninicillin, and 500 μg/ml streptomycin in crosses with streptomycin-sensitive prototrophic donors (Table 1). Colonies growing on the selective plates were counted 3-4 days later and were purified by restreaking at least twice on nonselective KB medium. Purified clones were stored on KB slants until further tests.

Characterization of R^+ transconjugants and confirmation of recipient phenotype.—Carbenicillin resistant clones were examined for inheritance of antibiotic resistance either by replica plating, or by the serial dilution method described previously. Identification of clones as recipients was made by observing the colony type, growth, and fluorescence characteristics, and by the following tests when applicable: (i) oxidase reaction (19), the recipients were negative (some *P. syringae* isolates were weakly positive, whereas *P. aeruginosa* donors were strongly positive); (ii) hypersensitivity reaction on tobacco (19), (*P. aeruginosa* strains were negative, whereas the plant pathogens were positive); (iii) pathogenicity on appropriate hosts (19); (iv) reaction towards phages E79, G101, D3, F116, and PRR1 and phage 2, [the first four phages lysed only *P. aeruginosa,* PRR1 was specific for strains harboring the RP1 factor (25); and phage 2 was specific for *P. phaseolicola*]; (v) growth at 42 C (only *P. aeruginosa* grew at this temperature).

Detection of penicillinase activity in colonies.—Penicillinase production was detected by two methods. In the starch-iodine test (26), approximately 10^8 to 10^9 cells were placed on starch-agar medium supplemented with 500 μg/ml carbenicillin. The petri dishes were then flooded with approximately 2 ml of iodine-potassium iodine solution and turned a dark blue. Penicillinase production was indicated by the development of a clear zone around the cells due to decolorization of the starch-iodine complex by the acid produced as a result of antibiotic hydrolysis. The second method was the Novick-Richmond modified N-phenyl-l-naphthylamine-azo-*o*-carboxybenzene (PNCB) test (24). The cells were streaked and grown on 0.3 CY medium for 2 to 4 days. The cultures were dried at 30 C, and the agar surface was flooded with 1.5 ml of a 0.25% (w/v) PNCB solution in N,N-dimethylformamide with 6% (v/v) 1.0 N NaOH (24). The dishes were again dried and then flooded with 0.1 to 0.2 M penicillin G solution. The acid released upon hydrolysis of the penicillin changed the acid-base indicator (PNCB) from yellow to purple.

Phage sensitivity tests.—Sensitivity to phage PRR1 and to phage 2 was tested by the double agar layer technique (1). A loopful of phage suspension was spotted on the top layer containing the test bacterium. The plates were incubated at 30 C and examined for lysis after 24 hours. Sensitivity to E79, D3, F116, and G101 was tested by cross-streaking a loopful of phage and of bacterial suspension on KB.

RESULTS.—*Genetic transfer of R18-1 and R91-1.*—Carbenicillin resistance specified by R18-1 was transferred from PA08 (R18-1) to a variety of plant pathogenic pseudomonads, including *P. phaseolicola* (7/8 isolates tested), *P. syringae* (5/5), *P. glycinea* (2/3), and *P. pisi* (1/1), and to the saprophytes *P. putida* (1/1), and *P. fluorescens* (1/1). Transfer frequencies differed

according to recipient (Table 2), but were generally between 10^{-6} to 2×10^{-3} R^+ recipients/donor cell. *Pseudomonas savastanoi* and *P. solanacearum* (three isolates in each case) were also tested for recipient ability against PA08 (R18-1), but with negative results.

In matings with PA08 (R91-1) donor transfer of carbenicillin resistance occurred to *P. pisi, P. syringae* (5/5), *P. fluorescens,* and *P. putida,* but not to any of the other recipients (Table 2). Transfer frequencies were much lower for R91-1 than for R18-1 in the same recipient strains (3×10^{-7} to 2×10^{-5}).

Genetic transfer of RP1.—Transfer of carbenicillin resistance carried by RP1 occurred from *P. aeruginosa* JC9006 (RP1) to *P. phaseolicola* (3/6), *P. glycinea* (2/2), and *P. syringae* (1/3) (Table 2). RP1-specified carbenicillin resistance was also transferred from *P. aeruginosa* 1822 to streptomycin resistant *P. phaseolicola* strains (Table 2). Co-inheritance of tetracycline,

TABLE 1. Origin and genetic properties of strains, plasmids, and phages used in experiments to test for conjugative transfer of P-group R factors to plant pathogenic *Pseudomonas* spp

Strain	Relevant genetic markers[a]		Origin and other properties
	R factor	Chromosomal genes	
P. aeruginosa			
PA08 (R18-1)	R18-1 *(amp)*	*met*-28, *ilv*-200, *str*^{r}	B. W. Holloway, FP$^-$
PA08 (R91-1)	R91-1 *(amp)*	*met*-28, *ilv*-200, *str*^{r}	B. W. Holloway, FP$^-$
1822	RP1 *(amp, neo, kan, tet)*	prototroph	E. J. L. Lowbury, obtained through B. W. Holloway
JC9006		*ade*$^-$	A. J. Clark, FP$^-$
JC9006 (RP1)	RP1 *(amp, neo, kan, tet)*	*ade*$^-$	MATERIALS AND METHODS
PAT404		*his*-404, *str*-1100	B. W. Holloway, FP2
P. phaseolicola			
HB32		prototroph	M. N. Schroth
HB33		prototroph	M. N. Schroth
HB36		prototroph	M. N. Schroth
HB3601		*met*-3	N. J. Panopoulos
HB3602		*his*$^-$, *leu*$^-$	N. J. Panopoulos
HB3600		*str*^{r}	N. J. Panopoulos
HB3604		*trp*-33, *str*^{r}	N. J. Panopoulos
HB39		prototroph	N. J. Panopoulos
HB41		prototroph	N. J. Panopoulos
HB45		prototroph	N. J. Panopoulos
R2QHB		prototroph	A. K. Vidaver, produces bacteriocin (38)
P. glycinea			
PG1		prototroph	M. N. Schroth
PG13		prototroph	M. N. Schroth
PG1271		prototroph	A. K. Vidaver, produces bacteriocin (38)
P. syringae			
Ps10		prototroph	A. K. Vidaver, produces bacteriocin (38)
Ps13393		prototroph	A. K. Vidaver, lysogenic (38)
Ps281		prototroph	A. K. Vidaver, lysogenic (38)
I6		prototroph	A. K. Vidaver (38)
S7		prototroph	M. N. Schroth
S54		prototroph	M. N. Schroth
P. marginalis 4		prototroph	M. N. Schroth
P. pisi 10		prototroph	M. N. Schroth, non-fluorescent
P. putida 110 (Stanier's)		prototroph	M. N. Schroth
P. fluorescens 20		prototroph	M. N. Schroth
Phage 2			E. Billing, specific for *P. phaseolicola*
Phage PRR1			R. H. Olsen, specific for RP1-harboring strains (25)
Phages D3, F116, G101, E79			J. Pemperton, specific for *P. aeruginosa*

[a]Genotype symbols conform to the rules of Demerec et al. (9). *amp, neo, kan, tet, str*^{r}: resistance to penicillins, neomycin, kanamycin, tetracycline and streptomycin, respectively; *met, ilv, ade, his, leu, trp:* requirement for methionine, isoleucine plus valine, adenine, histidine, leucine, and tryptophan, respectively; FP: lacking the sex factor FP; FP2: sex factor.

neomycin, and kanamycin resistance was demonstrated in all cases (Table 3). Transfer frequencies varied from 2×10^{-5} to 2×10^{-2} R^+ recipients/donor cell, which are noticeably higher than those obtained with R18-1 or R91-1 in the same recipient isolates (Table 2).

Characteristics of R^+ transconjugants.—Acquisition of R18-1, R91-1, and RP1 conferred on the recipients the capacity to produce penicillinase. Resistance to neomycin, kanamycin, and tetracycline remained unchanged in R18-1 and R91-1 recipients but increased 10 to 100 times in recipients of RP1 (Table 3). Recipients of the RP1 factor also became sensitive to phage PRR1 (Table 3), which is specific for strains that carry the plasmid (25). However, some RP1 recipients, such as *P. syringae* Ps13393 (RP1), *P. glycinea* PG1 (RP1), and PG1271 (RP1) were resistant to the phage. PRR1-resistant colonies of PRR1-sensitive transconjugants lost all four resistance determinants as well as the capacity to produce penicillinase. None of the following properties was changed as a result of R-factor inheritance: oxidase reaction, ability to induce hypersensitivity reaction on tobacco, reaction to phages F116, G101, E79, and D3 of *P. aeruginosa* or to phage 2 of *P. phaseolicola,* and pathogenicity (tested only in *P. phaseolicola, P. glycinea,* and *P. marginalis* R^+ transconjugants).

Stability and segregation of R factors in the transconjugants.—Carbenicillin resistance and penicillinase production were stable in most recipients grown in nonantibiotic media (Table 4). On occasion, however, spontaneous segregants appeared which had lost the capacity to produce penicillinase as well as the other antibiotic resistance properties which were associated with it. Although the frequency of segregation was low in most strains (0 to 3%, Table 4), R18-1 occasionally segregated at high frequency in *P. phaseolicola* HB36 (12%) and in one of its auxotrophic mutants (7%). Segregation frequencies were increased up to 94% when the bacteria were grown in the presence of SDS (Table 4) or upon long storage of slant cultures in the refrigerator.

Pseudomonas phaseolicola HB3600 (RP1) was examined for dissociation of the various resistance determinants from each other by replica plating. Of 600 colonies screened, one lost the *neo, kan,* and *tet* markers, whereas two others lost the *neo* and *kan* markers. All three retained the *amp* gene.

Donor ability of R^+ transconjugants.—Acquisition of R18-1 and RP1 by recipient strains was usually, but not invariably, associated with their subsequent ability to act as donors when crossed with recipients known to be R-accepting. RP1-harboring strains transferred all four resistance determinants, whereas strains harboring R18-1 transferred only carbenicillin resistance. The transfer frequency varied with the donor/recipient pair (Table 5).

Mechanism of transfer.—To establish whether or not the transfer of the R factors occurred by conjugation, rather than by transduction or transformation, cell-free culture filtrates were obtained by centrifugation and filtration (0.45-μm Millipore filter) from log-phase cultures of PA08 (R18-1) and strain 1822. No carbenicillin-resistant colonies were obtained when these filtrates were mixed in equal volume with *P. phaseolicola* HB33 recipient. In a separate experiment, log-phase PA08 (R18-1) cells were preincubated with 1,000 units/ml DNAase for 20 minutes prior to mixing. DNAase treatment was continued also during conjugation. No difference was found between DNAase-treated and nontreated control cultures in the frequency of transfer of carbenicillin resistance. These experiments, therefore, exclude transduction and transformation as mechanisms of transfer of antibiotic resistance from these donors.

TABLE 2. Host range and transfer frequencies of R18-1, R91-1, and RP1 to recipient *Pseudomonas phaseolicola, P. glycinea, P. syringae,* and *P. pisi.* Frequencies were calculated on the number of donor bacteria added at the start of the experiment and were based on the transfer of carbenicillin resistance from *P. aeruginosa* donors

Recipient	Donor		
	PA08 (R18-1)	PA08 (R91-1)	JC9006 (RP1)
P. phaseolicola			
HB32	9×10^{-5}	nt[a]	nt
HB33	1×10^{-4}	nd[b]	nt
HB36	1×10^{-4}	nd	nt
HB3600	nt	nt	2×10^{-5}
HB39	7×10^{-5}	nd	2×10^{-3}
HB41	5×10^{-4}	nd	nt
HB45	2×10^{-6}	nd	5×10^{-4}
R2QHB	2×10^{-4}	nd	nt
P. glycinea			
PG1	4×10^{-4}	nd	3×10^{-3}
PG13	1×10^{-6}	nd	nt
PG1271	nd	nd	5×10^{-3}
P. syringae			
Ps13393	2×10^{-3}	9×10^{-6}	2×10^{-2}
Ps281	3×10^{-4}	2×10^{-5}	nd
Ps10	2×10^{-4}	3×10^{-7}	nt
I6	2×10^{-5}	2×10^{-6}	nt
S7	6×10^{-6}	nd	nt
S54	1×10^{-5}	3×10^{-6}	nt
P. pisi 10	1×10^{-3}	5×10^{-5}	nt
P. marginalis 4	4×10^{-4}	nt	nt
P. putida 110	1×10^{-4}	6×10^{-6}	nt
P. fluorescens 20	8×10^{-4}	5×10^{-6}	nt

[a] nt = not tested.

[b] nd = not detected under the experimental conditions; $< 2 \times 10^{-8}$, R^+.

Kinetics and optimal conditions for transfer.—The time-course of transfer of carbenicillin resistance carried by R18-1 was examined in interrupted mating experiments. Interruption was accomplished by transferring the donor-recipient mixture to a test tube and vibrating in an Omnimix vibrator for 20 seconds. The kinetics of transfer showed linearity through the first hour of conjugation (Fig. 1). However, the number of transconjugants continued to increase at least through the first 3 to 4 hours.

Frequency of transfer of carbenicillin resistance from a given donor to a given recipient was variable between experiments. The transfer frequencies reported in Tables 1 through 6 were the highest ones recorded for the particular donor/recipient pair. The sources of the variation were not investigated in detail, but included (presumably) different time of incubation, variation in

the total cell density and the ratio of donor to recipient cells, presence or absence of nutrients that permitted some residual growth on the selective plates, and growth stage of the donor culture. Variation in the concentration of carbenicillin on the selective plates (200, 500, and 1,000 μg/ml) did not affect the recovery of transconjugants in test crosses between PA08 (R18-1) and HB36 (other crosses were not investigated in this respect). Plating serial dilutions of a mated cell mixture resulted in a disproportionate decrease of the number of transconjugants recovered on the plate. Washing the cells thoroughly before plating did not correct this inconsistency, thus ruling out the carry-over of penicillinase as the cause. It is possible that cells conjugated again on the plates. It should be noted that all (30/30) transconjugants picked from undiluted platings yielded stable penicillinase-producing clones after purification on nonselective medium, thus ruling out

TABLE 3. Antibiotic resistance, penicillinase production, and sensitivity to RP1-specific phage in R^+ and R^- strains of *Pseudomonas phaseolicola, P. glycinea, P. syringae,* and *P. aeruginosa*. The levels reported are the minimal inhibitory concentrations (MIC) determined as outlined in Materials and Methods

Strain	MIC values				Sensitivity to phage PRR1
	Carbenicillin	Neomycin	Kanamycin	Tetracycline	
P. aeruginosa					
PA08 (R18-1)	10,000	10.0	50.0	200.0	R
PA08 (R91-1)	10,000	10.0	50.0	200.0	
1822	10,000	>500.0	>500.0	400.0	
JC9006	50	0.5	0.5	25.0	R
JC9006 (RP1)	10,000	5.0	5.0	200.0	S
P. phaseolicola					
HB33	25	0.3[c]	0.3[c]	2.5	R
HB33 (R18-1)	10,000	0.3[c]	0.3	≤1.0	R
HB33 (R18-1$^-$), cured	200	0.5	0.5	≤1.0	
HB36	100	0.5	0.5	2.5	R
HB36 (R18-1)	10,000	0.3[c]	0.3[c]	1.0	R
HB3604	50	0.5	0.5	2.5	R
HB3604 (RP1)	10,000	5.0	5.0	25.0	S
HB3604 (RP1$^-$), cured[a]	50	0.5	0.5	2.5	R
HB39	25	0.3[c]	0.3[c]	2.5	R
HB39 (R18-1)	>4,000	0.3[c]	0.3[c]	≤1.0	R
HB39 (RP1)	10,000	≤2.5	25.0	50.0	S
HB45	50	≤0.1	≤0.1	2.5	R
HB45 (R18-1)	>4,000	≤0.1	≤0.1	≤1.0	R
HB45 (RP1)	>4,000	5.0	5.0	100.0	S
R2QHB	100	1.0	1.0	2.5	R
R2QHB (R18-1)	10,000	0.3[c]	0.3[c]	≤1.0	R
P. glycinea					
PG1	100	0.3[c]	0.1	2.5	R
PG1 (RP1)	>4,000	5.0	25.0	>100.0	S
PG1271	50	0.1	0.1	2.5	R
PG1271 (RP1)	>4,000	5.0	10.0	200.0	R
P. syringae					
Ps13393	200	0.5	0.5	5.0	R
Ps13393 (RP1)	10,000	2.5	12.5	200.0	R
Ps281 (R18-1)	R[b]	S[b]	nt	S	
Ps281 (R91-1)	R	S	nt	S	
Ps13393 (R18-1)	R	S	nt	S	
Ps13393 (R91-1)	R	S	nt	S	
Ps281	S	S	nt	S	
Ps13393	S	S	nt	S	
P. marginalis 4	S	nt	nt	nt	
P. marginalis 4 (R18-1)	R	nt	nt	nt	
P. putida 110	S	S	nt	S	
P. putida 110 (R18-1)	R	S	nt	S	
P. fluorescens 20	S	nt	nt	nt	
P. fluorescens 20 (R18-1)	R	nt	nt	nt	

[a] A colony selected for resistance to phage PRR1.

[b] Sensitive (S) and resistant (R) in disk assay to 10,000 μg/ml carbenicillin, 2,000 μg/ml neomycin, and 3,000 μg/ml tetracycline on the filter paper disk.

[c] The value 0.3 is rounded from 0.25.

TABLE 4. Segregation of penicillinase-negative clones in *Pseudomonas phaseolicola, P. syringae,* and *P. glycinea* R^+ transconjugants[a]

Strain	Penicillinase-negative segregants			
	Spontaneous		In presence of 0.1 to 1.02 SDS[b]	
	Colonies screened (no.)	Segregation (%)	Colonies screened (no.)	Segregation (%)
HB3601 (R18-1)	321	7.0	nt	...
HB36 (R18-1)	349	13.0	850	94.0
HB3600 (RP1)	164	0.6	nt	...
HB39 (RP1)	60	0.0	400	0.0
HB39 (R18-1)	nt	...	89	40.0
HB45 (R18-1)	nt	...	211	5.0
HB45 (RP1)	900	0.0	555	0.6
HB33 (R18-1)	1,050	3.0	283	27.0
R2QHB (R18-1)	nt	...	235	6.0
PG1 (RP1)	700	0.0	999	5.0
PG1271 (RP1)	500	0.0	568	0.0
Ps13393 (RP1)	221	0.0	200	0.0

[a]Cultures containing 10^1 to 10^4 cells/ml were grown in liquid complex medium with shaking. As soon as the cultures turned turbid, the cells were plated on 0.3 CY medium. The colonies were screened for penicillinase production by the PNCB test.
[b]SDS = sodium dodecyl sulfate.

TABLE 5. Donor ability of R^+ *Pseudomonas phaseolicola, P. glycinea,* and *P. syringae* transconjugants[a]

Donor	Recipient	Transfer frequency (carbenicillin-resistant transconjugants per donor)
R2QHB (R18-1)	JC9006	6×10^{-2}
HB45 (R18-1)	JC9006	1×10^{-3}
HB45 (RP1)	JC9006	1×10^{-6}
HB36 (R18-1)	JC9006	1×10^{-2}
HB33 (R18-1)	JC9006	1×10^{-2}
PG1 (RP1)	JC9006	3×10^{-7}
Ps13393 (RP1)	JC9006	7×10^{-7}
HB3602 (R18-1)	HB41	1×10^{-2}
HB3602 (R18-1)	HB33	1×10^{-2}
HB3602 (R18-1)	HB36	2×10^{-3}
HB3602 (R18-1)	HB45	2×10^{-4}
HB3604 (RP1)	Ps13393	5×10^{-4}
HB3600 (RP1)	PAT404	1×10^{-3}
HB39 (RP1)	JC9006	...[b]
PG1271 (RP1)	JC9006	...[b]

[a]Frequencies given represent transfer of carbenicillin resistance carried by the R factor.
[b]Nondetectable ($< 2 \times 10^{-8}$).

TABLE 6. Donor activity of *Pseudomonas aeruginosa* PA08 (R18-1) and 1822 cultures and culture filtrates in crosses with *P. phaseolicola* recipients[a]

Donor	Recipient	Frequency of transfer (R^+ transconjugants per donor cell)
P. aeruginosa PA08 (R18-1)		
whole culture	HB36	3×10^{-5}
cell-free filtrate	HB36	0 (5×10^{-8})[b]
whole culture	HB33	3×10^{-6}
whole culture + DNAase	HB33	3×10^{-6}
P. aeruginosa 1822		
whole culture	HB3604	4×10^{-5}
cell-free filtrate	HB3604	0 (2×10^{-7})[b]

[a]Log-phase cultures were filtered through 0.45-μm Millipore filters and the cell-free preparations were mixed with an equal volume of recipient culture. Whole-culture controls were also run in parallel, with a recipient/donor ratio of approximately 2. In the treatments involving DNAase the calls were suspended in Tris-maleate buffer, pH 5.5, the optimum pH for DNAase activity.
[b]Based on the number of cells per milliliter of whole culture.

syntrophism between donor and recipient as a reason for formation of colonies on the selective medium.

DISCUSSION.—The free exchange of extrachromosomal genetic elements among, and between, plant pathogenic and other bacteria are potentially significant from several standpoints. First, use of antibiotics to control plant diseases, although not extensive, is being practiced. At least in one important disease, fire blight of pears, antibiotic effectiveness has been nullified due to the development of resistant strains of the pathogen (21). Although R factors were not implicated in this case as the source of the resistance genes, such situations could emerge, should future use of antibiotics in plant disease control become more widespread. In addition to antibiotic resistance genes, plasmids are known which carry genes for resistance to inorganic salts, including mercuric salts (24), which were used as seed disinfectants. RP1 was shown to recombine with one such plasmid in *P. aeruginosa,* and to acquire a gene conferring resistance to mercuric chloride (32). Second, plasmids carrying determinants of plant pathogenicity and virulence, although not yet identified in natural strains of plant pathogenic bacteria, may conceivably exist. Recent reports (37, 39) have clearly implicated the essential role of a plasmid element as a genetic determinant of the tumor inducing ability of *Agrobacterium tumefaciens.* Such situations are known in human pathogens (23, 29, 31). Location of pathogenicity or virulence determinants on plasmids could be one of the reasons why plant pathogenic bacteria often become attenuated while being maintained in

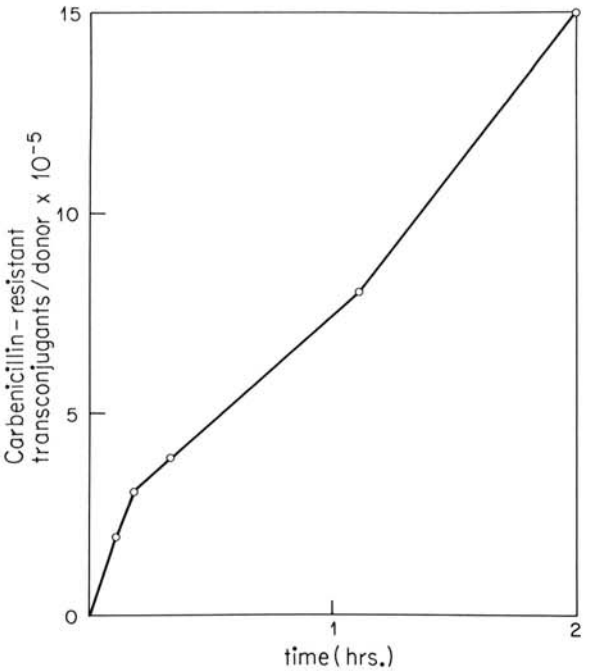

Fig. 1. Transfer of carbenicillin resistance from *Pseudomonas aeruginosa* PA08 (R18-1) to *P. phaseolicola* HB33 in liquid broth matings as a function of time. Stationary HB33 and log-phase PA08 (R18-1) cultures were mixed in test tubes and incubated statically at 30 C. At indicated times, cells were diluted 1:10, vibrated in an Omnimix vibrator for 20 seconds, and plated on minimal-glycerol-carbenicillin medium.

laboratory cultures. Pathogenic plasmids occurring naturally could furthermore obscure the epidemiology of bacterial diseases, if these were transmissible between the pathogens, and other saprophytic bacteria. Thirdly, isolation and identification of pathogenic plasmids, their manipulation by genetic and biochemical (3, 6) methods, and the remarkable versatility of bacterial gene transfer systems, could offer a new approach to the biological control of agricultural weeds.

This is the first documented report of genetic transfer by conjugation in the plant pathogenic pseudomonads and of free exchange of extrachromosomal elements between these organisms and a human pathogen. In the interspecific crosses involving RP1 donors, the genetic material being transferred was presumably extrachromosomal because this R factor has been identified by physical and genetic tests as an extrachromosomal genetic element in various bacteria (11). However, the physical state of R18-1 and R91-1 in *P. aeruginosa* PA08 is not known. R18-1 appears to be similar to RP1-1, the R factor studied by Ingram et al. (16) in *P. aeruginosa* Ps18 in that they both originated in *P. aeruginosa* 1822, confer transmissible high-level (> 10,000 μg/ml) resistance to carbenicillin only, and are poorly transmissible to the Enterobacteriaceae, in which they undergo spontaneous elimination at high frequency once introduced (16; Cho, et al., *unpublished*). Stable, penicillinase-producing clones have been isolated in *Escherichia coli* RP1-1 transconjugants and in *Erwinia* spp. R18-1 transconjugants by repeated subculturing in the presence of carbenicillin (see above references). In these clones, carbenicillin resistance is not transmissible. The stable RP1-1-harboring *E. coli* have the *amp* (carbenicillinase) gene inserted into the chromosome (28). In our studies, the RP1-specific phage (PRR1) did not lyse R18-1 transconjugants. Likewise, RP1-1-carrying *P. aeruginosa* is resistant to PRR1 lysis, although some increase in phage titer can be obtained in such strains (P. M. Bennett, *personal communication*). Other work (V. Stanisich, *communicated through* P. M. Bennett, *unpublished*) also indicated many similarities between R18-1 and RP1-1. Preliminary experiments in our laboratory also showed that RP1-1 is transferable to *P. phaseolicola* and *P. syringae,* but we have not determined the host range of this plasmid in all of our strains (27). The parallels between RP1-1 and R18-1 suggest that the two are closely related, or possibly identical, plasmids.

The relation between R18-1 and RP1 is not known. However, RP1-1 appears to be unrelated to RP1, since the two can coexist in one cell and do not show mutual transfer exclusion (V. Stanisich, *communicated through* P. M. Bennett). These two plasmids apparently share some DNA sequences in common but the extent of homology between them is not known, because the size of RP1-1 has not been determined. In addition, their β-lactamases cannot be distinguished by isoelectric focusing or other techniques (P. M. Bennett, *personal communication;* 16). The possibility that RP1-1 may be a fragment of RP1 (16) is now considered untenable, and the plasmid is believed to have originated by a recombination event between RP1 and another resident plasmid in the original host (P. M. Bennett, *personal communication*).

Regardless of its original state in the PA08 donor, R18-1 and R91-1 probably existed as plasmids at least in some of the plant pathogenic recipients. This is based on the spontaneous loss of penicillinase-producing ability, and presumably of the R factor itself, during storage or growth in the absence of carbenicillin, and the increased elimination frequency in the presence of SDS (11, 16, 30, 36). The large differences in the transfer frequency of R18-1 and RP1 from different transconjugants to *P. aeruginosa* JC9006 were not investigated in detail. Donor ability of RP1 transconjugants apparently did not correlate with susceptibility or resistance to phage PRR1.

The taxonomic significance of R-factor transfer, or the lack of it, in bacteria in general is uncertain (17, 23). Our limited survey of isolates further prevents us from making generalizations from our present results. However, certain trends were noted in our data. For example, none of seven *P. phaseolicola,* and of three *P. glycinea,* strains accepted the R91-1 factor; whereas, several *P. syringae* and the sole *P. pisi* strain did so. The frequency of transfer, however, was significantly lower for R91-1 as compared to R18-1 and RP1 in these strains. Neither R18-1 nor R91-1 was transferred to the three strains of *P. solanacearum* and of *P. savastanoi* that were tested (data not shown). It would be interesting to know whether or not these trends persist when a larger collection of strains of each species is examined.

The plasmids originally found in *P. aeruginosa* combine several interesting characteristics. They have a wide host range both within and outside of the

pseudomonads (7, 8, 25, 34, 35), ability to mobilize the bacterial chromosome (14, 33), and ability to recombine with other extrachromosomal elements (32) and with bacterial chromosomes (17, 28), resulting in the acquisition of new genes or the insertion of plasmid-borne genes onto the chromosome. Thus they could be utilized profitably in establishing conjugative transfer systems in different bacteria in which such systems have not yet been developed, such as the plant pathogenic pseudomonads. The transfer frequencies obtained in liquid broth matings were not very high, and efforts are now being made to determine methods of increasing these to levels which might permit detection of chromosomal transfer in various species.

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