Transmission of R Plasmids Among Xanthomonas spp. and Other Plant Pathogenic Bacteria

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ABSTRACT

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Plasmids RP4 and RK2 which specify resistance to carbenicillin, kanamycin, neomycin, and tetracycline were transmitted from *Escherichia coli* to *Xanthomonas vesicatoria*. Strains of *X. vesicatoria* that had acquired R plasmids were able to transmit them to other *Xanthomonas* spp., *Pseudomonas phaseolicola*, *Erwinia chrysanthemi*, and *Agrobacterium tumefaciens*, but not to *Corynebacterium michiganense*. The *Xanthomonas* plasmid transconjugants (ex-conjugants) exhibited multiple resistance to the above antibiotics and produced penicillinase. The stabilities of R plasmids in cells of xanthomonads differed with various cultures tested, ranging from 2 to 98% segregation during 10 days to 6 mo of storage. Different culture media affected frequencies of plasmid transfer. Acquisition of R plasmids had no effect on pathogenicity of *X. vesicatoria* transconjugants on tomato and pepper seedlings.

Additional key words: bacteria, plasmids, antibiotic resistance.

The study of bacterial antibiotic resistance factors (R plasmids) has continued steadily since they were discovered in *Shigella* strains in Japan about 16 yr ago (18). Only recently, however, have these investigations been broadened to include plant pathogenic bacteria. Group P plasmids have been transmitted to plant pathogenic *Pseudomonas* spp., to *Erwinia* spp. in vitro (4, 9, 22) and in planta (9), and to *Agrobacterium tumefaciens* (5, 14). Plasmids F'*lac* and R100*drd* also have been transmitted to certain *Erwinia* spp. (1, 3). Conjugative transfer systems have been established in a few species, and preliminary mapping of genes including one which determines virulence to plants, has been reported (2, 7, 12, 22, 23). Plasmids also have been shown to be essential genetic determinants for the tumor-inducing ability of *A. tumefaciens* (26, 27).

The transmission of antibiotic resistance plasmids among plant pathogenic bacteria may have important economic and epidemiological ramifications in the future. In California, streptomycin is being used on a wide scale for controlling fire blight of pear and a naturally occurring streptomycin-resistant strain(s) of *E. amylovora* was found in a pear orchard (17). At present there is no evidence that this resistance is controlled by a plasmid. The use of drug treatments for controlling bacterial plant diseases may become more widespread in the future in countries where no legislative restrictions exist. An understanding of the potential role of R plasmids in resistance to antibiotics in these bacteria may help establish and maintain effective treatment programs.

The successful transmission of antibiotic resistance among the phytopathogenic pseudomonads and erwinias prompted us to examine the plant pathogenic xanthomonads which were not included in previous studies. The presence of a slime layer in most *Xanthomonas* spp. raised doubts whether members of that genus could accept or transmit R plasmids. The host range of the plasmids RP4 and RK2 among *Xanthomonas* spp. is the subject of this report. An abstract of this work was published previously (13).

MATERIALS AND METHODS

Media.—Mineral broth (MB) contained 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, and 0.1 g $MgSO_4$ per liter H_2O. For succinate broth (SB) 5.0g of sodium succinate were added to MB and the pH was adjusted to 7.2. Difco nutrient broth (NB) also was used. Agar was added to the above at 20 g/liter to make Difco nutrient agar (NA). The (0.3 CY) medium (20), which was used for identification of penicillinase-producing clones, contained 0.3% casein hydrolysate and 0.3% yeast extract; sterile sodium glycerol phosphate was added to a final concentration of 0.01 M. The medium (YDC) used for storing cultures contained 10 g/liter yeast extract, 20 g/liter glucose, 20 g/liter $CaCO_3$, and 20 g/liter agar. Nutritional requirements of amino acid auxotrophs were added according to need at 0.3 mM concentrations.

Antibiotics.—Disodium carbenicillin was from Beecham-Massengill Pharmaceuticals, Bristol, TN 37620; penicillin G, chloramphenicol, and streptomycin sulfate from Calbiochem, San Diego, CA 92037; kanamycin sulfate from Eli Lilly and Co., Indianapolis, IN 46206; and tetracycline-HCl from Nutritional

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TABLE 1. Origin and genetic properties of bacterial strains and plasmids used in experiments to test for conjugative transfer of R factors to plant pathogenic bacteria[a]

Bacterial species and strains	R factor	Genetic markers	Origin
Escherichia coli			
J53		*pro*⁻[b], *met*⁻, F⁻	J. E. Beringer
J53 (RP4)	RP4 (*bla, neo, kan, tet*)[c]		J. E. Beringer
J53 (RK2)	RK2 (*bla, neo, kan, tet*)		J. E. Beringer
Pseudomonas aeruginosa			
PA08(R18-1)	R18-1 (*bla*)	*met*⁻, *ilv*⁻, FP⁻[d], Sm^r	B. W. Holloway
Xanthomonas vesicatoria			
XV5113		prototroph	M. Lai
XV5113-1		prototroph, Sm^r	XV5113
XB5113-3		*cys*⁻	M. Lai, XV5113
XV5092		prototroph	M. Lai
Xanthomonas malvacearum			
XM5121-1		prototroph, Sm^r	M. Lai, XM5121
XM5004-1		prototroph, Sm^r	M. Lai, XM5004
Xanthomonas pelargonii			
XP5123-1		prototroph, Sm^r	M. Lai, XP5123
Xanthomonas hederae			
XH5106-1		prototroph, Sm^r	M. Lai, XH5106
Xanthomonas diffenbachia			
XD5094-1		prototroph, Sm^r	M. Lai, XD5094
Xanthomonas corylina			
XC5032-1		prototroph, Sm^r	M. Lai, XC5032
Xanthomonas juglandis			
XJ5005-1		prototroph, Sm^r	M. Lai, SJ5005
XJ5026-1		prototroph, Sm^r	M. Lai, XJ5026
Xanthomonas incanae			
XI5010-1		prototroph, Sm^r	M. Lai, XI5010
Xanthomonas campestris			
XCm5002-1		prototroph, Sm^r	M. Lai, XCm5002
Pseudomonas phaseolicola			
PP4297		prototroph, Sm^r	N.J. Panopoulos, G52
Erwinia chrysanthemi			
EC3091		prototroph, Sm^r	M.N. Schroth, MNS138
Erwinia amylovora			
EA3088-1		prototroph, Sm^r	M. Lai, EA3088
EA3089-1		prototroph, Sm^r	M. Lai, EA3089
Agrobacterium tumefaciens			
AT1081-2		prototroph, Sm^r, Cm^r[e]	M. Lai, MT1081
Corynebacterium michiganense			
CM2046-2		prototroph, Sm^r, Cm^r	M. Lai, CM2046

[a]Phenotypic and genotypic designations are according to Demerec et al. (6) and Novick et al. (19) for chromosomal and plasmid traits, respectively.
[b]*pro, cys, met, ilv* = requirement for cysteine, methionine, and isoleucine plus valine, respectively.
[c]*bla, neo, kan, tet* = plasmid-encoded resistance to carbenicillin, neomycin, kanamycin, and tetracycline, respectively.
[d]F⁻, FP⁻ = lack of the sex factors F and FP, respectively.
[e]Sm^r, Cm^r = (chromosomal) resistance to streptomycin and chloramphenicol, respectively.

Biochemical Corp., Cleveland, OH 44128.

Bacterial strains.—*Escherichia coli* strains J53(RP4) and J53(RK2), each containing a plasmid specifying antibiotic resistance to carbenicillin (Cb), neomycin (Nm), kanamycin (Km), and tetracycline (Tc) and *Pseudomonas aeruginosa* PA08(R18-1), with a plasmid specifying resistance to carbenicillin (22), were used as initial donors. Original recipient strains were *Xanthomonas vesicatoria* XV5113 and XV5113-3. The source and identity of other recipient and donor strains are given in Table 1. Antibiotic-resistant mutants were isolated after exposure to a General Electric G15T8 15-W UV lamp at a distance of 0.3 m for 15-25 sec and plating on NA medium containing 500 μg/ml of the antibiotic.

Antibiotic resistance levels.—Potential recipient cultures were screened for resistance on streptomycin nutrient agar (SNA) containing 500 μg antibiotic/ml. Single-colony isolates were stored on YDC slants for further tests. All potential donor and recipient strains were cloned and tested for carbenicillin resistance on nutrient agar plus 500 μg carbenicillin (CNA) before use. Following purification, transconjugants were tested for multiple antibiotic resistance by introducing approximately 10^3 cells into a series of tubes containing 5 ml NB plus neomycin, kanamycin, or tetracycline, respectively, at two-fold dilutions ranging from 15 μg/ml to 100 μg/ml. Nutrient agar plus carbenicillin, neomycin, kanamycin, or tetracycline was used for quantitative tests. When chloramphenicol-resistant recipients were used in testing donor ability of transconjugants, the antibiotic was added to the proper medium at 100 μg/ml.

Mating procedure.—Donor and recipient cultures were grown overnight for 16-18 hr to late log phase in 10 ml of SB medium at room temperature in 125-ml Erlenmeyer flasks without shaking. Donor and recipient cultures were mixed (1:2, v/v) and 3 ml of the mixture was collected on a 0.45-μm Millipore filter disk which then was incubated on NA for up to 16 hr at room temperature. After the disk was washed in 2-4 ml of sterile water, serial dilutions were plated on media counterselective for the donor. Nutrient agar plus 500 μg/ml carbenicillin plus 500 μg/ml streptomycin (DAM) was used for streptomycin-sensitive donors. The latter plus chloramphenicol (100 μg/ml) was used in crosses between chloramphenicol-resistant recipients and chloramphenicol-sensitive donors. Presumptive transconjugants were counted 2-5 days after plating and were purified on selective medium. Purified clones were stored on YDC slants for further tests. Transfer frequencies (Tf) of conjugation tests were calculated from:

$$\text{Tf} = \frac{\text{Colony-forming units (CFU) of transconjugants per milliliter at time t}}{\text{CFU of donors per milliliter at time zero}}$$

Characterization of transconjugants and confirmation of recipient phenotype.—Carbenicillin-resistant clones were tested for antibiotic resistance by the serial dilution method described above. *Xanthomonas* transconjugants were distinguished from *E. coli* and *P. aeruginosa* donors by colony morphology, ability to hydrolyze starch, and ability to infect young tomato and pepper leaves. Inoculation was performed by dusting carborundum on the upper leaf surface and rubbing with a cotton swab dipped in a bacterial suspension (approximately 10^8 cells/ml). All transconjugants were assayed for penicillinase production by the Novick-Richmond modified N-phenyl-1-naphthyl-amine-azo-0-carboxybenzene (PNCB; Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, Long Island, NY 11514) test (20). Colonies grown for 1-2 days on 0.3 CY medium were tested by first flooding the plates with 1.5 ml of a 0.25% (w/v) PNCB solution prepared in N,N-dimethylformamide with 6% (v/v) 1.0 N NaOH added, drying, and flooding again with a 0.1 to 0.2 M solution of penicillin G. Hydrolysis of the penicillin changes the acid-base indicator (PNCB) from orange to purple, indicating penicillinase production.

RESULTS

Genetic transfer of RP4 and RK2 plasmids.—*Xanthomonas vesicatoria* XV5113 and its Cys$^-$ mutant XV5113-3 were originally crossed with *E. coli* J53(RP4) and J53(RK2) and *P. aeruginosa* PA08 (R18-1). Transmission frequencies, calculated as R$^+$ recipients per donor cell, differed with each recipient and each plasmid from 10^{-8} to 10^{-5} transconjugants/donor (Table 2). Multiple antibiotic resistance then was transmitted from XV5113(RP4) and/or XV5113(RK2) to a number of plant pathogenic xanthomonads: *X. vesicatoria* (two out of two isolates tested), *X. juglandis*

TABLE 2. Transmission frequencies of R plasmid-mediated carbenicillin resistance from *Pseudomonas aeruginosa* and *Escherichia coli* donors to *Xanthomonas vesicatoria*[a]

Recipient	Transmission frequencies from donor		
	P. aeruginosa PA08 (R18-1) Cb^r [Met$^+$,Ilv$^+$][b]	*E. coli* ECJ53 (RP4) Cb^r[Pro$^+$,Met$^+$][b]	*E. coli* ECJ53 (RK2) Cb^r [Pro$^+$,Met$^+$][b]
X. vesicatoria			
XV5113	$<10^{-8}$	1.2×10^{-5}	9.8×10^{-7}
XV5113-3	1×10^{-5}	2.6×10^{-5}	1.9×10^{-6}

[a]Transmission frequencies are expressed as the quotient of the number of colony forming units of transconjugants detected at sampling time divided by the number of colony forming units of donors added at time zero, after 16 hr of mating on nutrient agar.

[b]Markers used for selection of transconjugants. Those used for contraselection of donors are bracketed. Symbol Cb^r designates resistance to carbenicillin.

(two out of two and for each of *X. campestries*, *X. incanae*, *X. hederae*, *X. diffenbachiae*, and *X. pelargonii* (one out of one). Transmission frequencies differed for each mating, but ranged from 3.9×10^{-2} to 5.4×10^{-6} R^+ recipients per donor cell for the RP4 plasmid in 16-hr matings (Table 3). Matings between XV5113(RK2) and XV5113(RP4) donors and two naturally streptomycin-resistant strains of *E. amylovora* and one strain of *Cornyebacterium michiganense* were unsuccessful. Other strains of *E. amylovora* did, however, accept both RP4 and RK2 from *E. coli* (N. J. Panopoulos, *unpublished*) indicating strain specific transmission

Characteristics of R^+ transconjugants.—All R^+ transconjugants were tested for and showed penicillinase production. Multiple antibiotic resistance to kanamycin and neomycin (>250 to >1,000 μg/ml), and tetracycline (50 to 125 μg/ml) also was acquired by the recipients. Pathogenicity of XV5113 (ability to cause typical watersoaked lesions on pepper and tomato seedlings was unaffected by acquisition of either RK2 or RP4.

Most transconjugants retained their carbenicillin resistance and penicillinase production (88-97%) after storage on nonantibiotic medium (YDC) in the refrigerator for periods of up to 6 mo (Table 4). Some strains, however, showed substantial loss (up to 95%) in 10 days. Spontaneous segregation, detected as loss of the ability to produce penicillinase, was detectable in significant levels at 10 days of storage. Other antibiotic resistance properties associated with the plasmids also were lost in the segregants.

Optimal conditions for intrastrain transmission of plasmid RP4.—Mating periods from 1 to 16 hr were used in crosses between XV5113(RP4) and XV5113-1 to determine the kinetics of plasmid transmission. Matings were done on Millipore filter disks, which were washed in sterile water after the appropriate mating time and the suspension was plated serially on selective medium. Three series of tests were conducted: in the first, donor and recipient cultures were grown in NB, the Millipore filter disks were incubated on NA for up to 24 hr, and dilutions plated on DAM; in the second, donor and recipient cultures were grown in SB, with the remainder of the procedure as above; in the third, SB, SA, and DAM were used for each step, respectively. In the first series of tests, R^+ transconjugants were observed after a mating period of 4 hr (Fig. 1) and transmission frequency increased somewhat thereafter. In the second series of tests transconjugants were obtained at a frequency of 2.5×10^{-4} after 1 hr of mating. The frequency remained between 10^{-4} and 3.5×10^{-4} throughout the 16-hr period tested. In the third series the frequency of transconjugants was 5×10^{-3} after 1 hr of mating and increased to 2.3×10^{-2} to 10^{-1}

TABLE 3. Transmission frequencies of R plasmid mediated Cb-resistance from *Xanthomonas vesicatoria* transconjugants to other *Xanthomonas* spp., and non-xanthomonad plant pathogens[a]

Recipient	Donor	
	XV5113 (RP4) Cb^r [Sm^r][d] or XV5113-3 (RP4) Cb^r [Cys^+][d]	XV5113 (RK2) Cb^r [Sm^r][d] or XV5113-3 (RK2) Cb^r [Cys^+][d]
Xanthomonas spp.		
XP5123-1[b]	2.3×10^{-5}	5.4×10^{-6}
XM5121-1[b]	4.5×10^{-5}	5.7×10^{-4}
XV5113-1[b]	1.9×10^{-3}	6.5×10^{-4}
XH5106-1[b]	3.1×10^{-4}	3.3×10^{-4}
XC5032-1[b]	3.0×10^{-4}	6.6×10^{-4}
XJ5026-1[b]	1.5×10^{-2}	3.9×10^{-2}
XI5005-1[b]	1.9×10^{-3}	6.1×10^{-3}
XM5010-1[b]	7.5×10^{-4}	1.0×10^{-3}
XM5004-1[b]	3.2×10^{-3}	6.1×10^{-3}
Xcm5002-1[b]	2.6×10^{-5}	1.4×10^{-5}
XM5004-1[c]	...	1.5×10^{-4}
Pseudomonas phaseolicola		
PP4297[c]	1.3×10^{-4}	4.6×10^{-4}
Agrobacterium tumefaciens		
AT1081[c]	3.1×10^{-5}	3.6×10^{-5}
Erwinia chrysanthemi		
EC3091[c]	2.4×10^{-4}	5.6×10^{-6}
Corynebacterium michiganense		
CM2046[c]	$<10^{-8}$	$<10^{-8}$

[a]Transmission frequencies are expressed as the quotient of the number of transconjugants detected at sampling time divided by the number of donors added at time zero, after 16 hr of mating on nutrient agar.

[b]Donor strains were XV5113 (RP4) and XV5113 (RK2).

[c]Donor strains were XV5113-3 (RP4) and XV5113-3 (RK2). Selection was for Cb^r [Cys^+].

[d]Markers used for selection of transconjugants. Those used for contraselection of donors are bracketed. Symbol Cb^r designates resistance to carbenicillin.

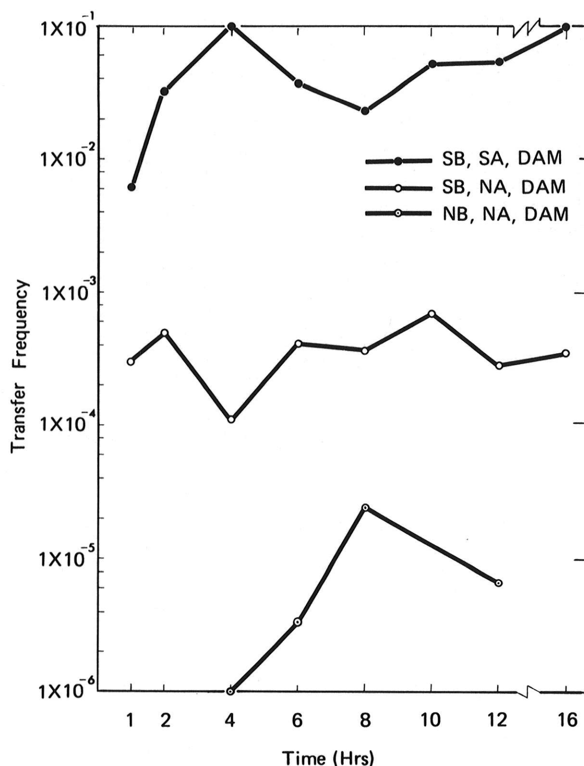

Fig. 1. Transmission of carbenicillin resistance from *Xanthomonas vesicatoria* XV5113 (RP4) to *X. vesicatoria* 5113-1 as a function of time. **Top line**: donor and recipient cultures grown in succinate broth (SB), matings conducted on succinate agar (SA), and transconjugants selected for on double antibiotic medium (DAM). **Middle line**: donor and recipient cultures grown in SB, matings conducted on nutrient agar (NA) and transconjugants selected for on DAM. **Bottom line**: donor and recipient cultures grown in nutrient broth (NB), matings conducted on NA, and transconjugants selected for on DAM.

thereafter. Visual observation indicated the donor and recipient cultures as well as the mating mixture on the Millipore filters had considerably less mucoid appearance in the second series of tests than in the first. Furthermore, mucoid appearance was considerably less pronounced in the third series of tests than in the other two.

DISCUSSION

These experiments are the first documentation of genetic transmission, presumably by conjugation, among species of *Xanthomonas*. The ability of the *Xanthomonas* donors to transmit multiple antibiotic resistance to a wide range of recipient strains was a further demonstration of the wide host range of RK2 and RP4 (4, 9, 21, 22). However, the relatively long mating periods required for genetic transmission suggest that problems will be encountered in the establishment of conjugative transfer systems within the genus.

Transmission frequencies for a given donor and recipient cross varied between experiments. Those reported in Tables 2 and 3 and in Fig. 1 were the highest obtained for each donor-recipient combination. Variations in cell density, donor/recipient ratio, mating time, growth stage of the cultures, recovery efficiency from the Millipore filter disks, and nutrient levels are all possible sources of variability in the transmission frequencies of a given mating.

Investigations are now being conducted to seek methods of increasing transmission frequencies. The P plasmids generally form weak mating pairs and this may account for the long mating periods required and the relatively low transmission frequency. Other reasons may be that fertility is repressed in the donor or that the recipient does not provide a good pilus attachment site(s). Thus, more strains need to be examined with the purpose of finding some with better donor or recipient ability. However, one variable which seemed to play a role in determining plasmid transmission frequencies between

TABLE 4. Spontaneous segregation of penicillinase-negative clones in *Xanthomonas* spp. transconjugants[a]

Strain	Penicillinase-negative segregants			
	10 days of storage		6 months of storage	
	Colonies screened (no.)	Segregation (%)	Colonies screened (no.)	Segregation (%)
XCm5002-1 (RP4)	98	3	414	92
XCm5002-1 (RK2)	70	7	992	87
XV5092-1 (RP4)	1,192	5	300	7
XV5092-1 (RK2)	148	16	319	8
XD5094-1 (RP4)	79	2	402	8
XD5094-1 (RK2)	945	5	398	4
XH5106-1 (RP4)	991	32	471	2
XH5106-1 (RK2)	324	5	399	5
XV5113-1 (RP4)[b]	121	95	426	96
XV5113-1 (RK2)[d]	NT	NT	446	98
XV5113-1 (RP4)[c]	30	33	NT	NT
XV5113-1 (RK2)[e]	248	54	NT	NT

[a]Penicillinase was determined by the N-phenyl-1-napthyl-amine-azo-0-carboxybenzene (PNCB) test.
[b,c]Different $RP4^+$ transconjugants.
[d,e]Different $RK2^+$ transconjugants.

XV5113(RP4) and XV5113 was the composition of the medium. It is unclear exactly what effect different carbon sources in the media have on the efficiency of plasmid transmission among these strains, but some hypotheses may be formulated from our results. At the outset of these experiments, all *Xanthomonas* cultures were grown in NB, incubated on NA, and plated on NA plus the appropriate antibiotics. The low frequency of plasmid transmission suggested that the mucoid slime layer typical of *Xanthomonas* spp. possibly inhibited transfer of the plasmids. A carbon source other than glucose generally reduced the amount of slime produced in culture. Thus, the procedure was modified in a variety of ways as already described. The results showed that transmission of the plasmid was much more efficient when the cells were grown in the absence of glucose. However, slime production during incubation of the Millipore filters on NA was evident and this may have inhibited conjugation and plasmid transmission to a certain extent. It should be noted, also, that transconjugant recovery on SA supplemented with carbenicillin plus streptomycin was very low.

The possibility of establishing genetic mapping systems in the plant pathogenic xanthomonads can be considered. Plasmids RK2 and RP4 as well as other P-group plasmids are known to mobilize the chromosomes of other bacteria at low or moderate frequencies (7, 10, 11, 12, 15, 24, 25). Recombinant DNA techniques also could be used for mapping as either complementary to or independent of classical chromosome mobilization. Both RP4 and RK2 have been reported to possess single EcoR1 sites and, thus, molecular vehicle properties (8, 16) and safe RK2 derivatives for DNA cloning are currently being constructed (D. R. Helinski, *personal communication*).

The stabilities of plasmids in xanthomonads grown in the medium without antibiotics varied with the different strains used (Table 4). However, these data do not permit conclusions as to the stability of these plasmids in plant tissues and other natural environments. This subject is currently under investigation.

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