Genetic Studies with Plasmid RP1 in Erwinia chrysanthemi Strains Pathogenic on Maize

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ABSTRACT

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Transfer of the wide-host-range plasmid, RP1, from *Escherichia coli* into four of twelve *Erwinia chrysanthemi* strains isolated from stalk rot of maize (EC_z) occurred at frequencies of 10^{-8} transconjugants per donor (TPD) or lower. However, transfer of RP1 from EC_z back to *E. coli* was more efficient at 10^{-4} TPD. The transfer frequency of RP1 was not increased by mating a RP1-cured recipient EC_z with an *E. coli* donor bearing RP1, acquired conjugatively from the same EC_z transconjugant strain. Transconjugants of EC_z became resistant to carbenicillin, neomycin, and tetracycline as well as sensitive to lysis by plasmid-specific phages PRR1 and PRD1. Transconjugants of EC_z also retained the ability to cause stalk rot symptoms on maize. Intraspecific transfer of RP1 from EC_z to three of six strains of *E. chrysanthemi* isolated from hosts other than maize was detected at frequencies of 10^{-8} to 10^{-5} TPD. Interstrain transfer of RP1 to eight strains of EC_z was successful once at a frequency of 10^{-8} TPD. Plasmid RP1 was transferred between mutants of a single EC_z strain at 10^{-5} TPD in vitro and 10^{-3} TPD in planta. Chromosomal transfer mediated by RP1 was detected in vitro among auxotrophic mutants of EC_z.

Additional key words: pathogenicity, in planta transfer, gene transfer, pseudotransconjugants.

Strains of *Erwinia chrysanthemi* Burkholder, McFadden and Dimock cause soft-rots of diverse dicotyledonous and monocotyledonous hosts (9, 31). Some *E. chrysanthemi* strains may be specialized in their ability to attack *Zea mays* L. (maize) and cause bacterial stalk rot (17, 21). They also are generally resistant to the cyclic hydroxamate 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) found in most American hybrid maize lines (4, 21). A role in host resistance has been postulated for DIMBOA (16). Furthermore, spontaneous variants of the maize pathogen (herein called EC_z) have yielded variants pathogenically less aggressive, morphologically altered, and nonmotile during routine maintainance of clones (Victoria, Kelman, and Lacy, *unpublished*). Some maize lines have manifested different levels of susceptibility, but not complete resistance to bacterial stalk rot (32). The specialization of the pathogen for its host, its natural variability, and degree of differential aggressiveness on maize make EC_z strains candidates for genetic analysis of pathogenicity.

Recently, several workers have developed recombination systems in *E. chrysanthemi* for studying the genetics of pathogenicity. Chatterjee and Starr (1) have described F'*lac*$^+$ mediated transfer of chromosomal elements and Guimares and Panopoulos (13) reported chromosome mobilization by the T plasmid, Rts1.

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This paper extends the number of plasmids expressing fertility functions in *E. chrysanthemi* strains, reports the characteristics of EC_z recipients and donors of P plasmids, and describes the expression of the plasmid functions in EC_z. Part of the information presented in this paper has been reported previously (22).

MATERIALS AND METHODS

Bacterial strains and phages.—The strains of bacteria, plasmids, and phages used in this research are listed in Table 1.

Media and maintainance of cultures.—All stock cultures were maintained on tetrazolium chloride agar (TZC) (18). Subcultures of EC_z were prepared for medium-term storage (1 to 6 mo) in tightly-capped vials at room temperature (21 to 24 C) by suspending bacterial cells in sterile distilled water until light turbidity was visible [about 10^7 colony-forming-units (cfu) per ml].

The basic media used were complete agar (CA), complete broth (CB), and minimal agar (MA) (23). The agar medium CPG had the same formulation as TZC except it did not contain tetrazolium chloride. Dilute one-tenth strength CA (1/10 CA) was prepared by adding that fraction of the usual amounts of casamino acids and yeast extract to the phosphate buffer in CA (23).

Sensitivity to antibiotics.—Tests for antibiotic sensitivity were conducted using antibiotic-impregnated disks (Baltimore Biological Laboratory, Inc., Cockeysville, MD 21030) applied to lawns of bacteria spread on CA. After incubation at 32 C, the plates were

examined for zones of inhibition (ZOI) around the disks.

Pathogenicity tests.—Plant material was prepared from 18- to 22-day-old hybrid maize seedlings derived by crossing University of Wisconsin public inbred lines W64A and W117. Pseudostem sections were prepared by cutting 1-cm sections from the "stalk" of young maize plants (which actually consists of tightly rolled, nonexpanded leaves above the apical bud). The sections were inoculated laterally by wounding with a toothpick contaminated with 48-hr colonial growth from CPG agar.

The procedures used for inoculation of detached leaves and whole plants have been described elsewhere (21, 32). Briefly, whole seedlings were inoculated by injecting 0.2 ml of aqueous suspensions of bacterial cells into the center of the pseudostem just above the apical meristem using a 0.315-mm diameter (30-gauge) needle. The wound was sealed with autoclaved petrolatum. Detached leaf segments were wounded by pressing with the tip of an Eppendorf 1 to 100 μliter micropipet (Brinkmann Instruments, Inc., Westbury, NY 11590). Then a 5-μliter droplet of bacterial suspension was placed on the wounded area. Incubation of inoculated plant material was carried out at 32 C and 100% relative humidity in the dark.

Selection of antibiotic-resistant mutants.—Spontaneous mutants were selected from cells suspended in CA supplemented with either 100 μg/ml rifampin, 100 μg/ml nalidixic acid, or 250 μg/ml

TABLE 1. Origins and phenotypes of bacteria, plasmids, and phage utilized in genetic studies of plasmid RP1 in *Erwinia chrysanthemi* strains pathogenic on maize

Strain	Phenotype	Origin and/or host	Source
Escherichia coli (Migula) Castellani & Chalmers 1919			
L-127	Arg^-, Leu^-/Cb^r,Nm^r,Tc^r	$RP1^+$ transconjugant constructed from X-705	G. H. Lacy
L-227	Arg^-,His^-,Leu^-,Pro^-, Thr^-,Sm^r/Cb^r,Nm^r,Tc^r	$RP4^+$ transconjugant	C. Boucher
X-705	Arg^-,Leu^-,Sm^r	induced mutations	R. Curtiss, III
X-705R$^+$	Arg^-,Leu^-,Sm^r/Cb^r, Nm^r,Tc^r	$RP1^+$ transconjugant constructed by X-705 × L-233	G. H. Lacy
Erwinia carotovora var. *carotovora* Dye 1969			
SR-53	Prototroph	carrot, Vermont	A. Kelman
L-231	Prototroph/Cb^r,Nm^r, Tc^r	$RP4^+$ transconjugant	M. C. M. Perombelon
Erwinia chrysanthemi Burkholder, McFadden & Dimock 1953 (from nonmaize hosts)			
D-52	Prototroph	*Chrysanthemum* sp., New York	R. S. Dickey
D-249	Prototroph	*Dracena* sp., Florida	R. S. Dickey
D-290	Prototroph	*Syngonium* sp., Florida	R. S. Dickey
D-362	Prototroph	banana (*Musa paradisiaca*) Honduras	R. S. Dickey
D-378	Prototroph	*Dieffenbachia* sp., Honduras	R. S. Dickey
D-600	Prototroph	sweet potato (*Ipomoea batatas*) Georgia	R. S. Dickey
(isolated from maize EC_z)			
L-188	Prototroph	nonmotile variant of SR-120	G. H. Lacy
L-196	$Pur\text{-}2^-$	NTG induced mutant of L-188	G. H. Lacy
L-211	$Pur\text{-}4^-$,$Trp\text{-}1^{-b}$	NTG induced mutant of L-188	G. H. Lacy
L-230	$His\text{-}1^-$,$Met\text{-}1^-$,$Sm^{r\,b}$	NTG induced (His^-,Met^-) and spontaneous mutations (Sm^r) of L-188	G. H. Lacy
L-233	Prototroph/Cb^r,Nm^r, Tc^r	$RP1^+$ transconjugant constructed from L-127 × SR-78	G. H. Lacy
L-234	Prototroph	L-233 spontaneously cured of RP1	G. H. Lacy
L-235	Na^r	Spontaneous mutant of L-234	G. H. Lacy
L-238	$Ilv\text{-}1^-$,Na^r	NTG mutant (Ilv^-) of L-188	G. H. Lacy
L-239	$Ilv\text{-}1^-$, Na^r/Cb^r, Nm^r, Tc^r	$RP1^+$ transconjugant constructed from L-238 × SR-120SR	G. H. Lacy
SR-57	Prototroph	maize, Egypt	K. A. Sabet
SR-59	Prototroph	maize, North Carolina	A. Kelman
SR-61	Prototroph	maize, Wisconsin	A. Kelman
SR-78	Prototroph	maize, Wisconsin	A. Kelman
SR-79	Prototroph	maize, Wisconsin	A. Kelman
SR-91	Prototroph	maize, India	M. M. Payak

(Continued)

TABLE 1. (continued)

Strain	Phenotype	Origin and/or host	Source
SR-94B	Prototroph	maize, North Carolina	A. Kelman
SR-120	Prototroph	maize, Hawaii	A. C. Hayward
SR-120S	Sm^r	Spontaneous mutant of SR-120	G. H. Lacy
SR-120SR	$Sm^r/Cb^r,Nm^r,Tc^r$	$RP1^+$ transconjugant constructed from L-127 × SR-120S	G. H. Lacy
SR-140	Prototroph	maize, Italy	U. Mazzucchi
SR-142	Prototroph	maize, Italy	U. Mazzucchi
Pseudomonas aeruginosa (Schroeter)Migula 1900			
PAO-25	$Met^-/Cb^r,Nm^r,Tc^r$	$R68\text{-}45^+$ transconjugant	B. W. Holloway
PAO-1670	$Pur^-,Leu^-,Rf^r/Cb^r$, Nm^r,Tc^r	$R6886^+$ transconjugant	B. W. Holloway
PYO 69/9169	$Prototroph/Cb^r,Nm^r,Tc^r$	$R9169^+$ transconjugant	E. J. L. Lowbury
Pseudomonas glycinea Coerper 1910[c]			
L-162	Arg^-,His^-,Met^-, $Sm^r/Cb^r,Nm^r,Tc^r$	$R6886^+$ transconjugant	G. H. Lacy
Phage			
PRD1	Lytic for P plasmid transconjugants		C. Boucher
PRR1	Lytic for P plasmid transconjugants		R. H. Olsen

[a]Abbreviations for antibiotics are: carbenicillin = Cb, neomycin = Nm, tetracycline = Tc, rifampin = Rf, streptomycin = Sm, and nalidixic acid = Na. Other abbreviations are according to Demerec et al. 1966 J. Gen. Microbiol. 50:1-14 (7).

[b]Double mutants of EC_z were recovered from separate rounds of mutagenesis.

[c]Doudoroff and Palleroni 1974 (Pages 217-243 *in* Bergey's Manual of Determinative Bacteriology, 8th ed. (8) have reduced *P. glycinea* to synonomy with *P. syringae* van Hall, however in this paper the former designation has been retained without intending any taxonomic clarification.

streptomycin sulfate. The plates were incubated at 28 C for 72 to 96 hr. Suspected mutants were restreaked on the same antibiotic-containing medium and then on TZC plates. Confirmation of the identity of the mutant and wild-type was made by comparing colonial morphology on TZC by the reflected light technique of Eigelsbach, et al. (10).

Phage sensitivity.—Sensitivity to phage PRR1 and PRD1 was demonstrated by inoculating a plate of CA with a single streak of the bacterial strain to be tested. A loopful of the phage suspension [10^8 to 10^{14} plaque-forming units (pfu) per ml] was spotted on the streaked portion of the plate. Lysis was detected by clearing in the otherwise confluent growth along the streak after 18 to 24 hr of incubation at 32 C.

Recovery of transconjugants.—Donor and recipient cells were prepared in CB by shaker incubation (140 rpm at 28 C) until the late log or early stationary phase was reached. Antibiotic selection was maintained to prevent loss of RP1 from donor cells by adding 500 μg/ml carbenicillin to the broth. Mating procedures and recovery of transconjugants were carried out in two ways:

Haas and Holloway's technique.—A drop containing recipient cells from a dense water suspension (10^8 to 10^9 cfu/ml) was placed on a CA plate. A second drop with donor cells was placed on the first drop. After incubation for 4, 8, or 12 hr, a wire loop was drawn through the growth, then streaked on selective media for isolation of transconjugants (14).

Rifampin counter selection.—Rifampin-resistant (to 100 μg/ml) recipient cells were spread on 1/10 CA (10^8 to 10^9 cfu per plate) with rifampin-sensitive donor cells. The plates were incubated 8 hr at 28 C. Then the mating cells were suspended in 2 ml of sterile water and the transconjugants were selected by suspending 1.0, 0.1, and 0.01 ml volumes of the cell suspension in CA prepared with 100 μg/ml rifampin and 10 μg/ml tetracycline. Donor populations were quantitated by either differential media [MA plus 250 μg/ml streptomycin and 20 μg/ml arginine and leucine for L-127 and King's Medium B (19) for PAO-25] or by sib culture for L-233. The frequency of plasmid transfer was recorded as the number of presumptive transconjugants per donor recovered (or TPD) after mating.

In planta plasmid transfers.—In planta transfer of RP1 was detected among washed suspensions of donors and recipients (10^8 cfu/ml) which were injected separately (in 0.1-ml volumes) into 21-day-old maize plants. After incubation at 32 C for 5 hr, the pseudostem (crown to expanding whorl) was removed, cut into 1-cm lengths and blended in 21 ml of water (Waring Blendor 700B, Winstead, CT 06098). The blended tissue was poured through sterile cheesecloth to remove large pieces of plant debris. Dilutions of the filtrate were plated on selective media.

Recombination experiments.—Overnight cultures of donors and recipients were incubated in the same manner as in the plasmid transfer experiments, and placed

together as droplets on a nitrocellulose membrane support (0.45 μm pore size) resting on a dry filter paper. The CB droplet containing donors and recipients (0.1 ml each of 10^9 cfu/ml) diffused through the membrane and into the filter leaving the cells in close contact on the surface. The membrane was transferred to a plate of 1/10 CA and incubated 8 hr at 32 C. The cells were resuspended from the membrane in 6 ml of water by vigorous agitation. Dilutions were plated on a selective medium. Recombinant frequencies were reported as the number of prototrophs or presumptive recombinants per donor (PPD).

RESULTS

Transfer of P plasmids to Erwinia chrysanthemi strains.—Two *E. chrysanthemi* strains, EC_z SR-78 and EC_z SR-120S, isolated from maize, were mated with *Escherichia coli* L-127 (RP1) on CA. In each case, transconjugants appeared after prolonged incubation (up to seven days) of the mating mixtures streaked on TZC supplemented with 500 μg/ml carbenicillin (TZC+Cb). Screening for transconjugants among the donors on TZC+Cb was facilitated by their colonial appearance since the donor, L-127 (RP1), formed white colonies, whereas both recipient EC_z strains produced red colonies.

Rifampin counterselection of donors indicated generally low or nondetectable transconjugant frequencies (Table 2). The donors included *E. coli* L-127 (RP1), EC_z strain L-233 (RP1), and *Pseudomonas aeruginosa* PAO-25 (R68-45). The recipients consisted of rifampin-resistant mutants of nine EC_z strains and six *E. chrysanthemi* strains isolated from nonmaize hosts. The plasmid RP1 was successfully transferred from L-127 to EC_z strains SR-79*rif* and SR-91*rif* as well as the nonmaize strain D-52*rif*. Intraspecific transfer of RP1 from L-233 was successful with three additional strains, EC_z SR-78*rif* and the nonmaize strains SR-249*rif* and D-600*rif*. No transconjugants were detected from matings with PAO-25 (R68-45) as the donor. The frequencies of recovery of transconjugants were low (about 10^{-8}TPD) except in one intrastrain mating (L-233 to SR-78*rif*) and one intergeneric mating (L-127 to D-52*rif*), in which $>10^{-5}$TPD were recovered.

Other bacterial strains with P plasmids also were used as donors. However, no transconjugants were detected when *P. aeruginosa* PAO-1670 (R6886) was mated with nine EC_z strains, when *P. aeruginosa* PYO 69/9169 (R9169) was mated with three EC_z strains, when *P. glycinea* L-162 (R6886) was mated with four EC_z strains, when *E. coli* L-227 (RP4) was mated with two EC_z strains, or when *E. carotovora* var. *carotovora* SR-44 (RP4) was mated with two EC_z strains.

Confirmation of plasmid transfer.—Confirmation of RP1 transfer to SR-78 (the RP1 transconjugant was designated L-233) was made by demonstrating: (i) that resistance to multiple antibiotics (carbenicillin, neomycin, and tetracycline) had been acquired simultaneously, (ii) that this multiple antibiotic resistance could be transferred to *E. coli* X-705, at frequencies greater than 10^{-4} TPD, and (iii) that the P plasmid-specific phages PRR1 and PRD1 (27, 28) could cause lysis. Transfer of RP1 to other EC_z strains was confirmed by the first two criteria above.

Characterization of the RP1 transconjugant, L-233.—*Erwinia chrysanthemi* L-233 was compared with SR-78 and *E. carotovora* var. *carotovora* SR-53 for pathogenicity on whole maize plants, detached leaves, and pseudostem sections. Strain L-233 produced rotting on the detached leaves and pseudostem sections in a manner similar to SR-78. *Erwinia carotovora* var. *carotovora* SR-53 caused no rotting on leaves or whole plants, but did cause a delayed rot (in 48 to 72 hr) on pseudostem sections. Strains SR-78 and L-233 produced

TABLE 2. Transfer of P plasmids to rifampin-resistant mutants (*rif*) of *Erwinia chrysanthemi* strains isolated from maize (EC_z) and nonmaize hosts

Recipient *E. chrysanthemi* strains	Frequency of plasmid transfer (TPD) per donor strain		
	E. coli L-127 (RP1)	*Pseudomonas aeruginosa* PAO-25 (R68-45)	*E. chrysanthemi* L-233 (RP1)
EC_z strains:			
SR-57*rif*	ND[a]	ND	ND
SR-59*rif*	ND	ND	ND
SR-61*rif*	ND	ND	ND
SR-78*rif*	ND	ND	4.2×10^{-5}
SR-79*rif*	1.0×10^{-8}	ND	1.8×10^{-8}
SR-91*rif*	3.8×10^{-9}	ND	ND
SR-94*Brif*	ND	ND	ND
SR-140*rif*	ND	ND	ND
SR-142*rif*	ND	ND	ND
Nonmaize strains			
D-52*rif*	1.2×10^{-5}	ND	8.9×10^{-7}
D-249*rif*	ND	ND	1.0×10^{-8}
D-290*rif*	ND	ND	ND
D-362*rif*	ND	ND	ND
D-378*rif*	ND	ND	ND
D-600*rif*	ND	ND	2.8×10^{-8}

[a]None detected. The limits of sensitivity for the matings were as follows: L-127 matings, 7.1×10^{-10} TPD; PAO-25 matings, 2.3×10^{-9} TDP; L-233 matings, 2.0×10^{-9} TPD.

rotting and lodging in five of six and four of six whole maize seedlings, respectively, in 72 hr and rotted leaf and pseudostem tissue in less than 24 hr. Strain L-233 was further compared with SR-78 by inoculation of API 50E biochemical tests (Analytab Inc., New York, NY 11514). No difference was noted between the strains on any of the 50 tests after 48 hr of incubation at 32 C.

Differences were noted in growth characteristics between the transconjugant and the wild-type EC_z. In CB shake cultures, L-233 produced flocculent growth, but SR-78 did not. On TZC plates, the 2- to 3-mm, irregular, slightly convex colonies of SR-78 contrasted with the small 1- to 2-mm, round, markedly convex colonies of L-233. Among colonies of L-233 on spread plates of TZC, 1 to 4% of the developing colonies resembled the colony-type produced by SR-78 rather than L-233. These SR-78-like colonies were found to have lost resistance to carbenicillin, neomycin, and tetracycline, the ability to transfer multiple antibiotic resistance, as well as sensitivity to PRD1 lysis. One of these spontaneous, RP1-cured clones was designated L-234 and retained for further study.

In planta plasmid transfer.—Genetic exchange among phytopathogens in planta under conditions of pathogenesis may be significant in the evolution of pathogenicity. In planta transfer of RP1 was detected in matings of EC_z L-233 with EC_z L-235 (a nalidixic acid-resistant mutant of L-234). In the first two in planta matings, bacteria from isolated colonies of both L-233 and L-235 were inoculated separately into the same wound in maize pseudostem sections with a toothpick. After 24 to 48 hr at 32 C, the rotting tissue was streaked on TZC + Cb plus 30 μg/ml nalidixic acid. Clones resistant to both carbenicillin and nalidixic acid were readily recovered.

The strains derived from SR-78 (L-233, L-234, and L-235) were sensitive to DIMBOA in vitro (21). Therefore, in a third experiment, the frequency of RP1 transfer in the cross L-233 × L-235 was compared in $DIMBOA^+$ (line 1552) and $DIMBOA^-$ (line 1455) maize lines derived by selfing (21) from Hamilton's original selection (15). The frequencies were 9.7×10^{-3} TPD in $DIMBOA^+$ and 3.6×10^{-3} TPD in $DIMBOA^-$ maize lines. These frequencies, however, were markedly higher than the transfer frequency of 3.4×10^{-5} TPD in the in vitro control for this experiment.

Characterization of L-234 as a recipient of RP1 from X-705R$^+$.—Possibly, L-234 or RP1, resident in L-233, had been modified to overcome entry exclusion (26). Therefore a quadratic check was used to evaluate the ability of L-234 to act as a recipient to RP1 from X-705R$^+$. In vitro matings were carried out for 4 hr on MA at 32 C in the following combinations: L-127 × SR-78, X-705R$^+$ × L-234, L-127 × L-234, and X-705R$^+$ × SR-78. The wild-type plasmid donor (L-127) and the wild-type recipient (SR-78) were the same as in the construction of the original transconjugant, L-233. The "compatible plasmid donor," X-705R$^+$, was constructed by transferring RP1 from L-233 into *E. coli* X-705 by conjugation. [Note that originally L-127 was constructed from X-705 (23)]. Strain L-234, derived from L-233 and spontaneously cured of RP1, was the "compatible plasmid recipient." The results of two experiments indicated that no transfer had occurred in any of the four matings among 10^8 recipients and 10^7 donors. This was consistent with the low transfer frequencies ($<10^{-9}$ TPD) routinely observed in the mating L-127 × SR-78. The plates were assayed for transconjugants by spreading on MA + Cb and MA + 10 μg/ml tetracycline (MA + Tc). Some colonies were noted on MA + Cb, but they did not maintain the transconjugant phenotype after subculture on the same medium. No colonies were detected on the tetracycline-fortified medium.

Pseudotransconjugants.—Carbenicillin was used in earlier experiments for selection of transconjugants and growth on MA as counter-selection against the auxotrophic RP1 donor L-127. This was based on tradition (23, 24, 28) and the observation that plasmid transfer frequency in *P. glycinea* was increased when carbenicillin, a cell wall-inhibitory antibiotic, was used rather than protein synthesis-inhibitory antibiotics such as tetracycline and neomycin as the selective agent for transconjugants (20). In several plasmid transfer experiments with EC_z, high frequencies of colonies appeared on carbenicillin-fortified selective media and presented typical transconjugant phenotypes. However, upon subculture, on the same selective medium, the "pseudotransconjugants" were unable to grow.

Intergeneric transfer of RP1 from EC_z L-233 to *E. coli* X-705 and intrastrain transfer of RP1 from EC_z L-233 to EC_z L-235 were accompanied by recovery of stable transconjugants. However, transfer of RP1 from *E. coli* L-127 to EC_z SR-78 or EC_z L-234 and from EC_z L-233 to EC_z SR-120 always was accompanied by the recovery of pseudotransconjugants. In a more detailed examination, individual colonies were tested for the ability to grow on subculture from the primary isolation medium onto the same selective medium. All of 40 colonies from each of the crosses L-233 × X-705 and L-233 × L-235 grew, but none of 36, 40, and 32 colonies from the crosses L-233 × SR-120, L-127 × SR-78, and L-127 × L-234 (respectively) were able to grow.

Examination of the constituents of pseudotransconjugant colonies from primary isolation plates indicated that they contained both donor and recipient cells (provided that there was no counterselection by a second antibiotic against the donor) and no transconjugants could be detected. Primary selective media fortified with tetracycline did not allow development of pseudotransconjugant colonies.

Gene transfer.—No native gene transfer system was detected in matings using SR-120 as donor to the double auxotroph L-230 with selections for recombinants to prototrophy and streptomycin counterselection against the donor. The number of prototrophs recovered at either locus was not increased significantly over the spontaneous reversion frequency at that specific locus (1×10^{-8} for *met* and 6×10^{-9} for *his*). Prototrophs for both *met* and *his* were not recovered.

Plasmid-mediated recombination was detected in matings between auxotrophic recipients L-196 (*pur*-2) and L-211 (*trp*-1 *pur*-1) and an auxotrophic donor L-239 (*ilv nal* RP1$^+$). The spontaneous mutation frequencies of the individual loci were *trp*-1, 9.7×10^{-9}; *pur*-1, less than 5×10^{-8}; *pur*-2, 1×10^{-8}; *ilv*, 4×10^{-9}; and *nal*, 2×10^{-9}. In two matings of RP1 donor L-239 with recipient L-196, 84 and 170 prototrophs were recovered on nonsupplemented MA. In the cross L-239 × L-211, 72 and 710 prototrophs

at both loci were recovered. The inoculum was approximately 10^7 cfu for both donors and recipients. No prototrophs were recovered from matings of L-238 with L-196 or L-211 in which RP1 was not a component. The mating of L-239 with L-211 was selected for further study.

In the third and fourth matings between L-239 and L-211 the frequency of prototrophy was 4.2×10^{-4} and 2.5×10^{-3} prototrophs per donor on nonsupplemented MA. In the fourth mating the frequency of prototrophy at each mutant locus of the recipient was compared with the frequency at both loci. The ratio of the frequency at one locus/two loci was 1.1 for *pur*-1, and 5.1 for *trp*-1. Prototrophic clones from several experiments were purified first on CA, then on MA and screened for resistance to nalidixic acid and carbenicillin as unselected markers. Among 12 recombinants, from a mating of L-239 × L-196, one was resistant to nalidixic acid. Among 55 prototrophs from two matings of L-239 × L-211, 37 were resistant to nalidixic acid. In all three matings, only one of the 67 clones that were screened was resistant to carbenicillin and could transfer resistance for the three antibiotics conferred by RP1 to *E. coli* X-705.

DISCUSSION

Introduction of P plasmids into EC_z was difficult. This was surprising since these plasmids have a wide host range among Gram-negative bacteria including cocci and obligate anaerobic forms (6, 28). Transfer was accomplished in four of twelve strains of EC_z tested, but in each case the frequency was less than 10^{-8} TPD. Three strains (D-52 *rif*, D-249 *rif*, and D-600 *rif*) of *E. chrysanthemi* from nonmaize hosts also accepted RP1. The frequency of RP1 transfer from L-127 into D-52 *rif* was reasonable for intergeneric transfer, but the frequency of transconjugants among D-249 *rif* and D-600 *rif* was low and similar to the frequency of RP1 transfer into EC_z. It was interesting to note that two of these nonmaize strains, D-249 *rif* and D-600 *rif*, also caused stalk rot symptoms when inoculated into whole plants, but D-52 *rif* did not (21, 32). Thus, no gross differences were noted between EC_z and nonmaize strains of *E. chrysanthemi* in the frequency of plasmid acceptance.

The studies reported here indicated a general resistance to plasmid aquisition by the recipient. The nature of this resistance is unknown. Some workers have reported low frequencies of transfer to individual strains (2, 28). Coplin and Stetak (3) reported a general failure to transfer RP1 plasmid from *E. coli* into *E. stewartii* (Smith) Dye. Only two of nineteen strains accepted the plasmid from *E. coli*.

Cho et al. (2) reported substantially higher frequencies of P plasmid transfer in their survey of the genus *Erwinia* including transfer frequencies as high as 2×10^{-3} and 3×10^{-4} TPD for strains of *E. chrysanthemi*. However, this discrepancy may be partially explained by how the number of donor cells were quantitated. In this study donor phenotypes were determined after mating, while in the other (2) they were determined before mating.

In *E. herbicola* (Lohnis) Dye, Gibbins et al. (12) reported that their strains were capable of acquiring plasmids intergenerically at reasonable frequencies (5×10^{-7} to 1×10^{-3} TPD), but were less efficient as plasmid donors to other bacteria including *E. herbicola* (frequencies usually less than 10^{-8} TPD). Gibbins et al. (12) indicated that failure to transfer resistance plasmids may have some epidemiological significance since P plasmids might be restricted from moving from *E. herbicola*, a common epiphyte of plants including pear, into *E. amylovora* (Burrill) Winslow, Broadhurst, Buchanan, Krumweide, Rogers and Smith, the causative agent of fire-blight. Likewise, failure of EC_z to act as a recipient to P plasmids may indicate a genetic isolation mechanism which could be an advantage for its evolution as a specialized pathogen.

Cryptic plasmid incompatibility was not a factor in the reluctance of EC_z SR-78 to act as a recipient to RP1. This was indirectly supported by the failure of X-705R$^+$ (a RP1$^+$ transconjugant of L-233) to transfer RP1 to L-234 (RP1-cured L-233) at an increased frequency. Therefore, the possibility of a host restriction mechanism must be entertained. That such a restriction was a contributing factor was shown in the higher frequency of RP1 transfer in the intrastrain crosses L-233 × SR-78 *rif* and L-233 × L-235.

Strain L-233, a RP1$^+$ transconjugant, rotted whole maize plants, detached leaves, and pseudostem sections. It may be a general rule that RP1 does not interfere with the ability of phytopathogens to cause disease since a RP1$^+$ transconjugant of *P. glycinea* also was able to cause susceptible as well as hypersensitive reactions on a range of bean and soybean hosts (23).

Plasmid transfer among EC_z strains occurred at an increased frequency in planta compared with in vitro matings. This also paralleled studies with *P. glycinea*, and again indicates that the transfer of plasmids among phytopathogens may be possible or even favored within or upon plants during pathogenesis. Further, an inhibitory compound, DIMBOA, did not affect the frequency of transfer when present in host tissue.

In this study, isolation of EC_z transconjugants was hampered by the pseudotransconjugant phenomenon Pseudotransconjugant colonies may be the result of syntrophism between the donor, protected from auxotrophic counterselection by the recipient, and the recipient, protected from antibiotic selection by the donor. The explanation for donor-protection of the recipient from the selective antibiotics may reside in the nature of the plasmid-borne antibiotic resistance. The β-lactmase responsible for resistance to carbenicillin is produced extracellularly (11), the phosphotransferase of RP4, a plasmid closely related to RP1, responsible for neomycin-kanamycin resistance (Julian Davies, *personal communication*), is periplasmic, while tetracycline resistance (not studied in P plasmids) usually is due to a mechanism (probably membrane-borne) preventing accumulation of inhibitory concentrations of the antibiotic (29). Perhaps pseudotransconjugants arise when intimate contact between donor and recipient cells allows enzymatic inactivation of the antibiotics. Tetracycline resistance, because of its nature, does not allow pseudotransconjugant colonies to develop.

Evidence for chromosomal gene transfer between auxotrophs of EC_z was based on (i) recovery of prototrophs from matings in which RP1 was a component at frequencies significantly higher than control matings (without RP1) and (ii) recovery of recombinants at an unselected marker (57% of the prototrophs also were resistant to nalidixic acid).

Plasmid RP1 was unstable in L-239 (up to 19% of the clones recovered were spontaneously cured) and could be maintained only with constant antibiotic selection. This situation was similar to the unstable donors generated in *P. aeruginosa* with plasmid R68-45 (14) and *P. glycinea* L-185 with plasmid R68* (25). However, no attempt was made to enhance the donor ability of RP1 in EC_z.

Transfer of chromosomal genes, at frequencies higher than plasmid transfer, has been encountered previously. The best-described system has been gene transfer from Hfr strains of *E. coli* in which the chromosomally-inserted fertility plasmid (F) is transferred after chromosomal markers (5). A similar phenomenon was observed in the transfer of chromosomal markers mediated by plasmids RP1 and R68 in *P. glycinea* (24). Possibly the position of RP1 with respect to the chromosomal markers transferred, has some influence on the frequency of RP1 transconjugants. Other explanations consistent with the data presented here might be (i) that RP1 transfers to recipients at high frequencies, but fails to establish itself in the EC_z recipients or (ii) that chromosomal transfer is mediated or enhanced by a cryptic, nonconjugative plasmid which is mobilized at high frequencies by RP1. The latter hypothesis may find some support since cryptic plasmids have been described in some EC_z strains (30).

In summary, EC_z strains are poor recipients but adequate donors of P plasmids. The presence of RP1 does not interfere with the ability of EC_z to be pathogenic on maize. Plasmid exchange between mutants of EC_z occurs readily in planta. The RP1 transconjugants of EC_z can mediate chromosomal recombination. In conclusion, EC_z may be investigated genetically by recombination mediated by RP1 and this system may be complimentary to the gene mapping system described by Chatterjee and Starr (1) in mapping distal markers.

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