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Plasmid-mediated transmission of chromosomal genes in *Pseudomonas glycinea**

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SUMMARY

Complementing diauxotrophic mutants of *P. glycinea* were mated in combination in which one or both mutants contained a resistance plasmid. The transfer of chromosmal markers was scored by the appearance of prototrophy at one or more of the auxotrophic loci. The resistance plasmid RP1 was equally or slightly more efficient than R6886 in the transmission of chromosomal genes.

1. INTRODUCTION

The genetics of phytopathogenic pseudomonads has been largely unexplored. (See Holloway, 1969; Holloway, Krishnapillai & Stanisich, 1971, for reviews on *Pseudomonas*.) However, genetic exchange has been achieved by transformation (Twiddy & Liu, 1972; Liu, 1973; Coplin, Sequeira & Hanson, 1974) and transduction (Garret & Crosse, 1963; Okabe & Goto, 1955) as has plasmid transfer by conjugation (Lacy & Leary, 1975; Panopoulos, Guimaraes, Cho & Schroth, 1975). Transfer of chromosomal genes by the latter process has yet to be demonstrated.

Phytopathogenic pseudomonads offer unique opportunities for research on the molecular basis of pathogenicity since they manifest great host–parasite specificity and distinct host reactions. Before such studies can be undertaken critically, an understanding of the inheritance of pathogenicity is required which is dependent upon development of conjugal gene transmission systems similar to those of *Escherichia coli* (Curtis, 1969) and *Pseudomonas aeruginosa* (Holloway *et al.* 1971).

Fertility functions of the incompatibility group P plasmids (Datta & Hedges, 1971) RP1 and R6886 are known from studies with *P. aeruginosa* (Stanisich & Holloway, 1969, 1971) and *E. coli* (Unger, personal communication). We previously transferred one of these plasmids (RP1) to *P. glycinea* and demonstrated transfer of the antibiotic resistance plasmid between *P. glycinea* and *P. phaseolicola* both *in vitro* and *in planta* (Lacy & Leary, 1975; Lacy, 1975).

* Doudoroff & Palleroni (1974) have provisionally reduced *P. glycinea*, *P. phaseolicola* and some other phytopathogens to synonomy with *P. syringae*. They caution, however, that these nomenspecies may be biotypes, pathotypes, varieties or independent species, but adequate comparative studies have not been carried out to determine their status. In this paper, the terms *P. glycinea* and *P. phaseolicola* have been retained without intending any taxonomic clarification.

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This paper describes the ability of RP1 and R6886 to mediate transfer of chromosomal genes between mutants of *P. glycinea.*

2. MATERIALS AND METHODS

(i) *Bacterial strains and plasmids*

These are listed in Table 1.

(ii) *Mutagenesis, mutant selection and isolation procedures*

Nitrosoguanidine (100 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in tris-malate buffer (pH 8·0) (Delic, Hopwood & Friend, 1970) was used as the mutagen. After mutagenesis, the cells were inoculated into complete broth (CB) (Lacy & Leary, 1975) and incubated overnight at 25 °C. The culture was washed by centrifugation, resuspended in nitrogen-free minimal broth (MB) (Lacy & Leary, 1975), and shaken for 4 h to force metabolic dormancy as measured by oxygen uptake on a Gilson differential respirometer (Model GR-20).

Table 1. *Origin of bacterial strains and mutants*

Bacterium	Strain	Genotype	Origin
Escherichia coli	L-127	*arg leu str* (F^-RP1)	a
Pseudomonas aeruginosa	PAO2609	*leu pur rif* (R6886)	b
P. glycinea R6	Pg-9	*met str*	c
P. glycinea R6	L-143	*leu met pro str*	d
P. glycinea R6	L-146	*his met arg str*	d
P. glycinea R6	L-147	*his met pur str*	d
P. glycinea R6	L-149	*met orn pro str*	d
P. glycinea R6	L-162	L-146 (R6886)	e
P. glycinea R6	L-163	L-146 (RP1)	f
P. glycinea R6	L-164	L-143 (RP1)	f

(*a*) Constructed by mating *E. coli* X-705 with *P. aeruginosa* PAT904 Rev 1. (Lacy & Leary, 1975).
(*b*) Received from V. A. Stanisich, Monash University, Clayton, Victoria, Australia 3168.
(*c*) Received from M. N. Schroth, University of California, Berkeley, California, U.S.A. 94720.
(*d*) Nitrosoguanidine mutants of Pg-9.
(*e*) Constructed by mating L-146 with PAO2609.
(*f*) Constructed by mating L-143 and L-146 with L-127.

Two basic selection procedures were used. The first was a modification of Ornston, Ornston & Chou's (1969) cyclic counter selection technique with D-cycloserine. The cells were centrifuged and resuspended in MB supplemented with any growth factors required by the prototroph plus 1000 μg/ml carbenicillin and incubated 8 h. Pelleted cells were osmotically shocked (Belser, personal communication) by agitation in sterile water for 60 sec, centrifuged, and resuspended in either CB or supplemented MB. Supplemented MB was used if a specific marker was being selected. The regimen of starvation and lysis alternating with out-growth was repeated twice.

The second selection procedure was a modification of the procedure described

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by Carhart & Hegeman (1974) and Carhart (personal communication). Centrifuged cells were resuspended in 1·8 ml of lysis medium at a density of 10^9 to 10^{10} colony forming units (cfu)/ml. At 6, 12 and 24 h after initiation of lysis, 0·1 ml of D-cyclo-serine (660 μg/0·1 ml) was added to a final concentration of 946 μg/ml. Twelve hours later the cells were osmotically shocked, centrifuged, and resuspended in CB for 8–12 h.

Isolation of mutant clones was made directly from minimal agar (MA) (Lacy & Leary, 1975). Prototrophic colonies which developed after 48 h of incubation were marked on the bottom of the Petri dishes. Wells, 5 mm in diameter, were cut in the agar near the centre of the plates and filled with either a solution of the specific compound (usually 20 mg/ml) or a solution of casamino acids and yeast extract (15 and 1·5 g respectively in 100 ml water). New colonies developing in the next 72 h were checked for their auxotrophic requirements by the method of Holliday (1956).

Exposure to nitrosoguanidine for 60 min allowed recovery of 8–10 per cent survivors. Quantitative comparisons between the modified Ornston *et al.* (1969) and Carhart & Hegeman (1974) D-cycloserine selection procedures were not made. However, the final ratio of auxotrophs to prototrophs ranged from 10^{-3} to 10^{-2} with both procedures.

(iii) *Mating and detection of recombinants*

Recipient and donor strains (Table 1) were cultured separately in CB at 25 °C on a reciprocal shaker for 14–16 h. The cell densities were adjusted turbidimetrically with CB to 10^8–10^9 cfu/ml and equal volumes of the two-cell suspensions mixed and incubated for 4 h in static culture.

To assay for recombinants, aliquots of appropriate dilutions of the mating suspensions in 0·85 % sodium chloride (SS) were spread on MA plus the appropriate nutrient or antibiotic supplements. Donor cells were selected on MA containing 5 μg/ml tetracycline hydrochloride and 10 μg/ml neomycin sulfate in addition to their individual growth requirements.

The lowest dilutions of the mating suspensions were pipetted into 45 mm Millipore filter assemblies containing 50 ml of SS. Cells were impacted on 0·45 μm pore-size filter membranes by suction and rinsed with additional volumes of SS to remove any residual CB. The membranes were transferred to appropriate agar media.

The spread plates and filter membranes were observed for colonial growth after 72 h at 25 °C. Recombinants were reported as the number of prototrophs per 10^9 cfu of donor cells in the mating suspension.

3. RESULTS

Recombinants to prototrophy at one or two loci were detected by colonial growth on MA when pairs of complementary, double-auxotrophic mutants of *P. glycinea*, in which one or both members contained either RP1 or R6886 as a resident plasmid,

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were mated (Table 2). Recombinants at one locus were recovered 8–18 times more frequently than recombinants at two loci.

In matings in which only one member of the mated pair had a resident plasmid (L-162 × L-143, L-163 × L-143), RP1 (in L-163) was twice as effective as R6886 in mediating transfer of genes for recombination at *leu* and *pro* or at *orn* and *pro*. In crosses using L-164 (RP1) as the donor, recombination at both *arg* and *his* occurred about 100-fold less frequently than at *his* or *pur*.

Table 2. *Summary of gene transmission experiments between auxotrophic mutants of* P. glycinea *Pg-9 mediated by the antibiotic resistance plasmids RP1 and R6886*

Mated isolates of *P. glycinea*		Prototrophs/ 10^9 donor cfu
L-162 *arg his* (R6886)	× L-143 *leu pro*	1293
	× L-149 *orn pro*	267
L-163 *arg his* (RP1)	× L-143 *leu pro*	2450
	× L-149 *orn pro*	440
L-164 *leu pro* (RP1)	× L-162 *arg his* (R6886)	12
	× L-163 *arg his* (RP1)	17
	× L-146 *arg his*	42
	× L-147 *his pur*	1626
L-143 *leu pro*	× L-147 *his pur*	0
L-146 *arg his*	× L-143 *leu pro*	4
	× L-149 *orn pro*	2

Reversion to prototrophy at any of the individual loci was negligible ($1{\cdot}0 \times 10^{-9}$ to $1{\cdot}0 \times 10^{-7}$) except for the *pro* marker ($2{\cdot}4 \times 10^{-5}$). Data from the control crosses, L-146 *arg his* × L-143 *leu pro* and L-146 × L-149 *orn pro* (Table 2), provides the only evidence for reversion to prototrophy at both loci.

In two matings, L-162 × L-164 and L-163 × L-164, both diauxotrophic mates contained plasmids. The numbers of prototrophic recombinants were reduced markedly (Table 2). Such reductions would be expected if plasmid entry-exclusion by another plasmid of the same incompatibility group was functioning in these crosses.

As previously described for RP1 *in vitro* and *in planta* (Lacy & Leary, 1975), R6886 is also transferred very effectively to recipient cells. Our observations indicated that 10^{-3} to 10^{-1} of the exconjugant recipient cells manifested the antibiotic resistance spectrum specified by the plasmid resident in the donor strain. Among the recombinants at either the *arg* or *his* locus from L-162 × L-143, 30–60 % demonstrated the plasmid's antibiotic resistance. However, 98 % of the recombinants at both loci had plasmid-conferred resistance. These percentages are based on 281 and 101 clones tested respectively.

4. DISCUSSION

These results demonstrate that transfer of chromosomal genes may be mediated by the *p* incompatibility group plasmids RP1 and R6886 in *P. glycinea*. The numbers of recombinants obtained compare favourably with those recorded by Stanisich &

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Holloway (1971) in *P. aeroginosa*. Their results indicated that R9169 (not used in this study) and R6886 were more effective than the *P. aeruginosa* fertility plasmid (FP2). Our results with *P. glycinea* suggest that RP1 is possibly twice as effective as R6886.

This demonstration of plasmid-mediated chromosome transfer in *P. glycinea* should now allow the construction of genomic maps by methods comparable to those used in *P. aeruginosa* (Loutit, 1969). The similarity between P^+ *P. glycinea* and F^+ *E. coli* in terms of the frequency of plasmid and chromosome transfer should also encourage the search for plasmid-integrated (Hfr) strains of the former. Such deratives have been constructed in *E. coli* using a variety of plasmids (Nishimura, Nishimura & Caro, 1973) including RP1 (Unger, personal communication). The availability of these strains would allow the genetic analysis of phenomena such as pathogenicity and host resistance among phytopathogens.

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