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Linkage Analysis of *Pseudomonas glycinea*

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The IncP-1 plasmid R68 and variants R68.45 and R68.185 were tested for their chromosome donor ability in a selected recipient of *Pseudomonas glycinea* PGR12. It was found that these variants did not express their selected characteristic of increased donor ability over that of R68 or R68.5, our commonly used donor plasmids. Coinheritance analysis of a variety of crosses provides evidence of a linkage group comprising 11 loci.

Phytopathogenic bacteria, such as the fluorescent pseudomonads, offer unique opportunities for the study of host-parasite interactions. We have chosen the soybean-*Pseudomonas glycinea* host-parasite system in which to study the genetics of pathogenesis. This phytopathogen manifests an intimate parasitic relationship with its host, demonstrates great host specificity, and produces distinct pathogenic versus hypersensitive resistance reactions in the host. This makes the assay for pathogenicity quick, simple, and straightforward.

Antibiotic resistance plasmids (R plasmids) have been instrumental in the development of conjugational systems in various procaryotes. Beringer and Hopwood (2), Johnston and Beringer (6), Meade and Signer (11), and Kondorosi et al. (7) have reported the ordering of genes on segments of *Rhizobium* species chromosomes by using R plasmids. Holloway has ordered genes by using natural sex factors and R plasmids in *P. aeruginosa* (5). We have shown previously that the IncP-1 plasmid R68 has the capacity to mediate chromosomal gene transfer in *P. glycinea* (9).

Chatterjee and Starr (3) mapped the chromosome of the soft-rot bacterium *Erwinia chrysanthemi* by integrating F'*lac*$^+$ into the chromosome forming an Hfr. They subsequently mapped a pectolytic enzyme implicated in pathogenicity between a histidine locus and a threonine locus.

We have developed a system, using the antibiotic resistance plasmid R68, R68.45, and a variant plasmid obtained in our laboratory for mapping auxotrophic and drug resistance markers, to produce a detailed chromosomal map of *P. glycinea*. We present here chromosome mapping studies of *P. glycinea* obtained by selection of prototrophic recombinants and analysis of coinheritance of unselected markers after conjugal transfer.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1.

Mutant isolation. Naturally occurring methionine mutants of *P. glycinea* Race 6 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (9) or ethyl methane sulfonate (Sigma Chemical Co., St. Louis, Mo.). After mutagenesis with 0.5% ethyl methane sulfonate for 5 h, the subsequent steps were essentially those described by Watson and Holloway (12). The incubation temperature was 25°C, the optimum growth temperature of *P. glycinea*. Surviving colonies were transferred to minimal agar supplemented with amino acid pools so as to identify the new auxotrophic mutations.

Tryptophan mutants were further defined by plating 10^8 colony-forming units onto minimal agar supplemented with indole or anthranilic acid by adding a few crystals of the chemical to the center of the plate. Mutants capable of growth with both anthranilic acid and indole are tryptophan mutants unable to make anthranilic synthetase and are designated *trpE*. Those mutants unable to utilize either chemical have lesions in the gene determining tryptophan synthetase $\beta 2$ subunit and were designated *trpB* (10). Mutants which grew with indole but did not grow with anthranilic acid were not identified at this time but are considered mutations of genes different from *trpB* and *trpE*.

Plate mating. One-tenth milliliter amounts of donor and of recipient cultures were mixed on supplemented minimal agar (8) to select for single locus recombination. Recombinants appeared during 3 to 5 days of incubation at 25°C. Donor and recipient cells plated separately and matings without plasmids served as controls. Transfer frequency represents the number of colonies that appeared on the mating plate per donor cell. Determination of which loci were coinherited with the selected marker was done by transferring the recombinant colonies onto appropriately supplemented minimal agar. The selected recombinants were routinely streaked to determine the stability of the prototrophs. Once we established their stability, such checks were performed only when new loci were involved.

The percent coinheritance of unselected markers was determined by plating recombinant colonies onto

minimal agar lacking another of the required nutrients and dividing the number of recombinant colonies which grew by the number of recombinants tested.

Plasmids. The IncP-1 antibiotic resistance plasmid R68 (8, 9) and a variant selected for increased chromosome donor ability, R68.185 (Lacy et al., Proc. Am. Phytopath. Soc., abstr. no. 86, 1976), were transferred to PGR202, and these strains were designated as PGR2020 and PGR2023. R68.45, the variant sex factor from *P. aeruginosa* (4), was also transferred into PGR202, and the strain was designated PGR2022.

In the cross PGR2020(R68) × PGR12, an Ilv^+ His^+ recombinant was selected and tested for donor ability. The selected recombinant was designated PGR2120(R68). PGR2120(R68) was crossed with a naturally cured strain of PGR2020. The resulting recombinants were tested for donor ability, and the one with the highest donor ability was designated PGR2021(R68.5). PGR2021(R68.5) has remained a consistently reliable donor. Additional donors [PGR1051(R68) and PGR1071(R68)] were also selected as recombinants that carry R68 and were used in subsequent matings.

TABLE 1. *Bacterial strains used*

Designation	Genotype[a]
PGR12	*met-1*[b] *his-1 ilv-1 trpE1 nalA1*[c]
PGR14	*met-1 his-1 ilv-1 trpB1*
PGR16	*met-1 his-1 ilv-1 trp-3*
PGR20	*met-1 his-1 ilv-1 trp-4 ser-1*
PGR21	*met-1 his-1 ilv-1 ser-2*
PGR105	*met-1 leu-1 lys-1 trpB3*
PGR107	*met-1 leu-1 phe-1 trpB2*
PGR1071(R68)	*met-1 phe-1*
PGR2020(R68)	*met-1 leu-1*
PGR2021(R68.5)	*met-1 leu-1*
PGR2022(R68.45)	*met-1 leu-1*
PGR2023(R68.185)	*met-1 leu-1*
PGR2120(R68)	*met-1 trpE1 nalA1*
PGR1051(R68)	*met-1 lys-1 nalA1*

[a] The following genotypic abbreviations have been used for requirements for: histidine: *his*; isoleucine/valine: *ilv*; leucine: *leu*; lysine: *lys*; phenylalanine: *phe*; serine: *ser*; tryptophan: *trp*.
[b] A naturally occurring methionine requirement.
[c] Nalidixic acid resistance >2 mg/ml.

RESULTS AND DISCUSSION

We have performed a number of three-factor crosses with different recipients, donors, and plasmids. The matings can best be characterized by using the recipients PGR12, PGR14, PGR16, PGR20, PGR105, and PGR107. The percents coinheritance of unselected markers are shown in Tables 2 through 4.

Donors containing the plasmid R68 or variants of this plasmid described above were mated to the recipient PGR12. The data (Table 2) indicate that in nearly all matings Ilv^+ recombinants were recovered at a slightly greater frequency than either His^+ or Trp^+ recombinants. When the relative donor abilities of the plasmid variants were compared, it was apparent that, although each of the presumed variants was originally selected for increased donor ability,

TABLE 3. *Coinheritance of unselected markers when PGR2021(R68.5) is used as donor*

Recipient	Selectd marker	% Coinheritance[a]			
		Ilv^+	His^+	Ser^+	Trp^+
PGR14	*ilv-1*		58	—[b]	16
	his-1	52		—	22
PGR16	*ilv-1*		58	—	14
	his-1	29		—	4
PGR20	*ilv-1*		56	36	20
	his-1	65		39	25
	ser-1	10	18		17
PGR21	*ilv-1*		77	60	—
	his-1	64		60	—
	ser-2	20	40		—

[a] Number of colonies tested varied according to number of recombinants available (100 or more).
[b] Not determined.

TABLE 2. *Comparison of frequency of transfer of selected markers and coinheritance of unselected markers when different plasmids are used as vehicles*

Donor	Selected marker	Transfer frequency[a]	% Coinheritance[b]		
			Ilv^+	His^+	Trp^+
PGR2020(R68)	*ilv-1*	5.7×10^{-7}		57	17
	his-1	1.2×10^{-7}	61		28
	trpE1	2.2×10^{-7}	14	21	
PGR2021(R68.5)	*ilv-1*	8.1×10^{-7}		77	39
	his-1	3.0×10^{-7}	32		8
	trpE1	2.4×10^{-7}	21	21	
PGR2022(R68.45)	*ilv-1*	4.8×10^{-7}		56	16
	his-1	2.0×10^{-8}	50		25
	trpE1	2.4×10^{-7}	30	28	
PGR2023(R68.185)	*ilv-1*	1.7×10^{-7}		100	44
	his-1	6.2×10^{-8}	60		33
	trpE1	5.2×10^{-8}	39	44	

[a] Transfer frequency is the number of recombinants per donor cell. The recipient strain was PGR12.
[b] Number of colonies tested varied according to number of recombinants available (100 or more).

TABLE 4. *Coinheritance of unselected markers in the crosses PGR1071(R68) × PGR105 and PGR1051(R68) × PGR107*

Recipient	Selected marker	% Coinheritance[a]				
		NalA	Leu$^+$	Lys$^+$	Trp$^+$	Phe$^+$
PGR105	*leu-1*	—[b]		21	12	—
	lys-1		3		8	—
	trpB3		2	18		—
PGR107	*leu-1*	0		—	0	0
	lys-1	82	0	—	1	
	trpB2	4	0	—		8

[a] Number of colonies tested varied according to number of recombinants available (100 or more).
[b] Not determined.

there is essentially no difference in the frequency of transfer of Ilv$^+$, His$^+$, or Trp$^+$ by the different plasmids.

The coinheritance data indicate that *his-1* and *ilv-1* are more closely linked than either *his-1* and *trpE1* or *ilv-1* and *trpE1*. In repeated matings, variations in the coinheritance of the markers being studied were seen. However, these variations never altered the relative position of the loci in the linkage group.

When tryptophan mutants other than *trpE1* were used in matings with PGR2021(R68.5), *trpB1*, *trp-3*, and *trp-4* markers could not be selected directly but still showed about the same linkage with *ilv-1* and *his-1* as did *trpE1* (Table 3).

In matings involving PGR105 and PGR107, *trpB2* and *trpB3* were selected and shown to be linked to *leu-1* and *lys-2*. Resistance to the antibiotic nalidixic acid (*nalA*) is linked closely to *phe-1* and is distant from *trpB3* and *leu-1*.

Based on the coinheritance data in Tables 2 through 4, we envision the linkage to be as described in Fig. 1.

Additional evidence that supports the abovementioned arrangement is the lack of segregation of middle markers when selecting for flanking markers (3). Segregation of the middle marker would require four crossing-over events, a phenomenon assumed to be rare (Table 5). For example, the gene order *ilv-1 his-1 trpE1* shown in Fig. 1 is consistent with the fact that the least frequent class of recombinants is that requiring four crossing-over events. Other sets of markers that were ordered this way were: *leu-1 ilv-4 his-2*; *ilv-1 his-1 ser-1*; *leu-1 lys-1 trpB3*; and *trpB2 phe-1 nalA1*.

When an auxotrophic marker in a donor strain

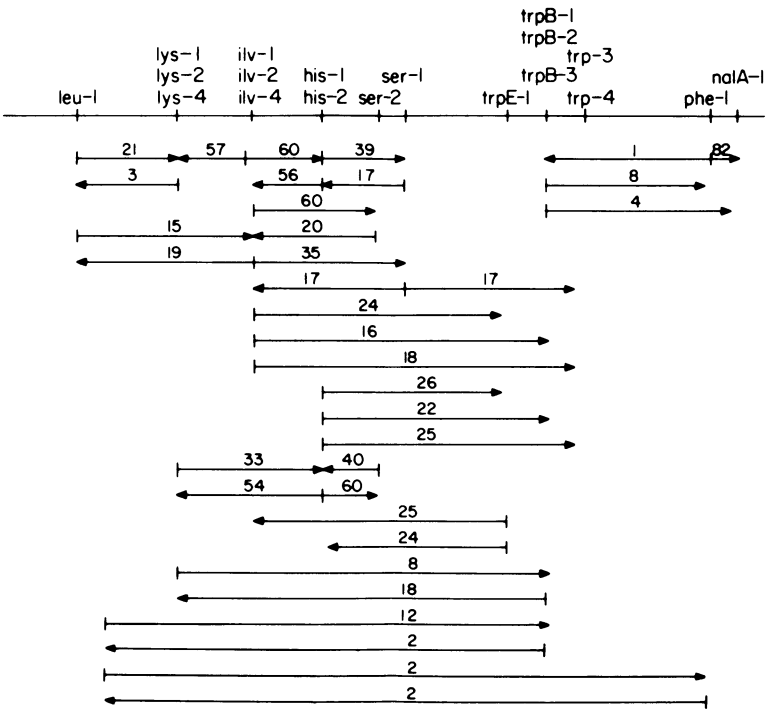

FIG. 1. *Linkage map of P. glycinea. Linkage values are expressed as the percentage of selected recombinants that coinherited the unselected marker, averaged over one to five crosses. Arrowheads indicate the unselected marker with the selected marker at the tail. Some loci and linkages are from crosses not discussed in the text.*

TABLE 5. *Analysis of three-factor crosses*

Selected[a] marker (no. tested)	Unselected marker	Frequency of recovery (%)
Cross I		
ilv-1$^+$ (679)	*his-1*$^+$ *trpE1*	46
	his-1 trpE1$^+$	1
	his-1$^+$ *trpE1*$^+$	16
	his-1 trpE1	37
Cross II		
ser-2$^+$ (57)	*ilv-1*$^+$ *his-1*	3
	ilv-1 his-1$^+$	14
	ilv-1$^+$ *his-1*$^+$	18
	ilv-1 his-1	65
Cross III		
ilv-1$^+$ (89)	*his-1*$^+$ *trp-3*	66
	his-1 trp-3$^+$	1
	his-1$^+$ *trp-3*$^+$	14
	his-1 trp-3	19
Cross IV		
his-1$^+$ (68)	*ser-1*$^+$ *trp-4*	13
	ser-1 trp-4$^+$	0
	ser-1$^+$ *trp-4*$^+$	25
	ser-1 trp-4	62

[a] PGR2021 was the donor strain in all crosses. PGR12, PGR21, PGR16, and PGR20 were recipient strains in crosses I through IV, respectively.

falls between a selected marker and a distal unselected marker it results in a lowering of coinheritance of the distal marker. We see evidence of this in the cross PGR1051(R68) × PGR107, where the coinheritance between *leu-1* and *trpB2* is reduced. The *lys-1* marker of the donor strain maps between these two loci and therefore would be expected to reduce the observed linkage.

The *trpB, trp-3,* and *trp-4* loci were placed near the distal end of the map based on the difficulty we experienced in direct selection for these markers plus the relatively low coinheritance of these loci with *leu-1*. Originally, none of these loci could be selected. However, each of the *trp* loci is coinherited with *ilv-1*. Recently, by constructing new donors PGR1051 and PGR1071, and using the recipients PGR105 and PGR107, we were able to select for *trpB* recombinants (Table 4). Therefore, it appears that there is an effect of the strains used, both donor and recipient, on the ability to obtain recombinants for the distal markers. Additional evidence for this is apparent when one considers that *trpB* is transferred at a high frequency when the recipient is not of the *met-1 his-1 ilv-1* genotype background.

R68.45 and R68.185 did not express their selected characteristic of enhanced chromosome donor ability. Kondorosi et al. (7) report that in *Rhizobium meliloti* R68.45 transfers genes near to the frequency reported in *P. aeruginosa* PAO, whereas Beringer and Hopwood (2) report frequencies in *R. leguminosarum* similar to our findings. Donor strain PGR2120(R68.5) was selected as a plasmid-containing recombinant from earlier matings and was shown to have increased donor ability in initial tests. This procedure follows that of Haas and Holloway (4) in selecting R68.45, which increased donor ability in *P. aeruginosa*. However, subsequent matings using this donor indicated that the increased donor ability originally selected for was not maintained.

We conclude from these findings that: (i) we have established a linkage group based on the transfer of linear segments of the chromosome; (ii) R68.45 does promote gene transfer in *P. glycinea* but not at increased frequencies; and (iii) *P. glycinea* lends itself to mapping, using conjugation and linkage analysis, which should greatly facilitate the positioning of chromosomal determinants of virulence and pathogenicity.

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