SHORT COMMUNICATION

An Indigenous System of Gene Transfer in the Plant Pathogen *Pseudomonas morsprunorum*

By JEFFERY ERRINGTON AND ALAN VIVIAN*

School of Biological Sciences, Thames Polytechnic, Wellington Street, London SE18 6PF

(Received 15 January 1981)

A fertility system has been discovered in the phytopathogen *Pseudomonas morsprunorum*. The recovery of recombinants carrying both selected and unselected genetic markers from crosses indicated that relatively large segments of bacterial chromosome may have been transferred. Fertility was restricted to particular combinations of strains. The system may represent an important means of genetic recombination in the natural environment.

INTRODUCTION

Pseudomonas morsprunorum is the causative organism of bacterial canker of cherry (*Prunus avium*) and plum (*Prunus domestica*) in England. Strains of *P. morsprunorum* show a high degree of specificity in nature and infect either cherry or plum (Crosse & Garrett, 1970). Cherry-specific strains are further subdivided into two distinct races, designated 1 and 2, on the basis of their specificity for particular cultivars (Freigoun & Crosse, 1975). Biochemical tests and phage typing are used to distinguish strains of *P. morsprunorum* from those of the closely related *P. syringae* (Garrett *et al.*, 1966). Race 2 isolates are further subdivided into three groups on the basis of pigment production, arbutin hydrolysis and phage typing (Garrett *et al.*, 1977). Until about 1970, most cherry-specific strains were of race 1 type: cherry cultivar Napoleon was susceptible to these strains whereas cultivar Roundel was relatively resistant. After 1970, race 2 strains began to appear and cultivar Roundel showed an increased susceptibility to this new race.

The genetic basis of the differences between these closely related strains of bacteria is clearly important both for an understanding of how they might have evolved and also for the more practical purpose of cultivar selection in relation to strain specificity. A prerequisite for genetic investigation is a system of gene transfer and in this paper we report the discovery of an indigenous system of gene transfer in a race 1 strain of *P. morsprunorum*.

METHODS

Bacterial strains. A race 1 strain of *Pseudomonas morsprunorum*, designated C28, was originally isolated at East Malling Research Station, Kent and was donated by Dr J. E. Crosse. The following auxotrophic mutations were obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine: *his-1*, *leu-1*, *nic-3* and *trp-1*. All other mutations were obtained after mutagenesis with ethyl methanesulphonate. All cultures were grown at 25 °C; stock cultures were maintained on slopes at 4 °C.

Media. Complete medium was Nutrient Glycerol Agar (NGA): this contained 0.8% (w/v) nutrient broth (Oxoid), 2.0% (w/v) glycerol and 2.0% (w/v) Bacto-agar (Difco). Minimal medium (MM) was Mineral Salts of Clowes & Hayes (1968) solidified with 2% (w/v) Bacto-agar (Difco) and supplemented where appropriate with amino acids (each at a final concentration of 50 µg ml⁻¹) and/or vitamins (1 µg ml⁻¹). Glucose was prepared as a 50% (w/v) solution, sterilized separately and added to MM at a final concentration of 0.5% (w/v). Liquid cultures

were grown in nutrient broth (NB; Oxoid). All media and supplements were sterilized by autoclaving at 121 °C for 15 min.

Mutagenesis. This was done by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methane-sulphonate, according to the methods of Fargie & Holloway (1965) for *P. aeruginosa* but omitting penicillin treatment.

Millipore mating technique. This was essentially as described by Towner & Vivian (1976). Equal volumes of 24 h aerated NB cultures containing about 5×10^8 cells ml⁻¹ were mixed and 3 ml of this mixture was drawn slowly through a Millipore filter (0.45 µm pore size, 25 mm diam.). The filter was placed on the surface of an NGA plate and incubated for 24 h. Following incubation the filter was transferred to a sterile container and the cells were resuspended in 3 ml quarter-strength Ringer's solution (Oxoid). This suspension was washed once before plating on appropriately supplemented MM.

Analysis of recombinants. Crosses were made between doubly-marked auxotrophic strains and recombinants were selected on four different media, each containing two auxotrophic supplements in non-parental combinations (Hopwood, 1959). Each medium selects for one marker in each parent and leaves the remaining two markers unselected. Recombinant colonies were tested for inheritance of unselected markers by replica plating to appropriately supplemented MM.

RESULTS AND DISCUSSION

Recombinant formation and analysis

Crosses were performed between derivative strains of C28, each carrying two genetic markers. An example of a typical cross is given in Table 1. The cross was plated to select for all four possible combinations of selectable markers. Recombinants were obtained on all four selections at frequencies of about 3.5×10^{-7} recombinants per recipient. Since these frequencies were considerably higher than the highest reversion frequency for a single marker (3.8×10^{-9} , for *gly-1*), the involvement of genetic recombination in crosses of this type was clearly indicated.

Recombinant colonies were tested for inheritance of unselected markers (Table 1*a*). The majority of recombinants carrying a single auxotrophic marker differed by only a single marker from the E221 parent, indicating that transfer was polarized, with E71 acting as a donor and E221 as a recipient. Similar polarity has been observed with 'recipient' strains carrying different auxotrophic markers, for example strains E10 (*his-1 nic-3*), E63 (*his-1 arg-3*), E65 (*his-1 phe-2*) and E66 (*his-1 ilv-2*), indicating that reversion of particular alleles is unlikely to be involved. Further, on the basis of a large number of crosses with different 'donor' strains, 'recipient' strains always behaved as 'recipients'.

The data in Table 1(*b*) show that the average coinheritance frequency of *ilv*⁺ when selection was made for *nic*⁺ was greater than the coinheritance frequency of *nic*⁺ when selecting *ilv*⁺. Furthermore, this asymmetry was independent of the counterselection. This implies that there may be a definite origin of transfer with *ilv-4* being transferred earlier than *nic-3*. The coinheritance frequencies indicate that *nic-3* and *ilv-4* are relatively close to one another and that neither *met-5* nor *trp-1* is closely linked to either of these two markers. In a further cross (data not shown) involving E71 as a 'donor' and a strain derived by recombination, E232 (*arg-1 leu-3 nic-3 ilv-4*), a similar asymmetry of inheritance of *ilv-4* and *nic-3* was observed. Out of 42 recombinants selected as *nic*⁺, 35 were also *ilv*⁺ (83 %); while 21 out of 37 recombinants selected as *ilv*⁺ were also *nic*⁺ (56 %).

It is possible to envisage prototrophic recombinants such as those obtained in Table 1 arising by reversion of the two markers involved in one or other parent, although control experiments indicate that this would occur at a frequency of 10^{-18} . However, the recovery of doubly-auxotrophic recombinants would require reversion at one locus followed by a forward mutation in a specific class of gene; the probability of this occurring would be even lower than that of obtaining prototrophic recombinants and is for all practical purposes considered impossible. In the course of some 50 crosses many doubly-auxotrophic strains, such as E201 (*his-1 leu-1*), E202 (*his-1 thi-3*), E205 (*nic-3 trp-1*) and E212 (*ilv-2 trp-1*), have been obtained, confirming the conclusion that true genetic recombination is involved and also that

Table 1. *Results and analysis of a typical cross: E71 (trp-1 met-5) × E221 (nic-3 ilv-4)*(a) *Frequencies of unselected genotypes*

Genotype class	Selected markers				Genotype frequencies (per 10 ⁸ recipients)			
	<i>met</i> ⁺ <i>ilv</i> ⁺	<i>met</i> ⁺ <i>nic</i> ⁺	<i>trp</i> ⁺ <i>ilv</i> ⁺	<i>trp</i> ⁺ <i>nic</i> ⁺				
<i>trp</i>	0	0	—	—	0	0	—	—
<i>ilv</i>	—	17	—	16	—	7.0	—	6.0
<i>met</i>	—	—	3	6	—	—	1.2	2.3
<i>nic</i>	47	—	37	—	17.6	—	14.7	—
<i>trp nic</i>	0	—	—	—	0	—	—	—
<i>trp ilv</i>	—	0	—	—	—	0	—	—
<i>met nic</i>	—	—	0	—	—	—	0	—
<i>met ilv</i>	—	—	—	0	—	—	—	0
+	53	67	51	59	19.8	27.4	20.3	22.2
Total	100	84	91	81				

—, Genotype classes which are not recoverable on the selective medium.

(b) *Coinheritance frequencies of selected and unselected donor markers**

Selected donor marker	Unselected donor marker	Counterselected marker	Coinheritance	
			Observed	%
<i>nic</i> ⁺	<i>ilv</i> ⁺	<i>trp</i>	65/81	80
	<i>ilv</i> ⁺	<i>met</i>	67/84	80
	<i>trp</i>	<i>met</i>	0/84	0
	<i>met</i>	<i>trp</i>	6/81	7
<i>ilv</i> ⁺	<i>nic</i> ⁺	<i>trp</i>	54/91	59
	<i>nic</i> ⁺	<i>met</i>	53/100	53
	<i>trp</i>	<i>met</i>	0/100	0
	<i>met</i>	<i>trp</i>	3/91	3

* Assuming polarized transfer: E71 as donor (see text).

relatively large fragments of donor chromosome may be transferred in the process. Such a system is considered most likely to involve conjugation.

An attempt was made to determine whether transformation might be responsible for the recombination observed. For example, in duplicate crosses between strain E71 (*trp-1 met-5*) and strain E10 (*his-1 nic-3*), one cross was treated with deoxyribonuclease both before and after incubation on the Millipore filter: there was no detectable difference in the frequencies of *his*⁺ or *nic*⁺ recombinants between the two crosses. A significant reduction in recombinant frequency in the treated cross would have been expected if transformation were responsible for the fertility, although the possibility that transformation may have occurred during incubation on the Millipore filter cannot be excluded.

At present it seems unlikely that transduction is involved because of the large numbers of markers (about half of those used in crosses) which show linkage and the indications that relatively large fragments of chromosome may be transferred. However, the possibility that phage might be involved has not been excluded.

The nature of 'donors' and 'recipients'

There is some fertility between 'donor' strains, but recombinants have not been obtained from crosses between 'recipient' strains; however, it should be said that since most of the 'recipient' strains only differ from each other by single markers, it has not been possible to test this extensively. Preliminary results indicate that 'donor' and 'recipient' ability segregates amongst recombinants. This may enable us to obtain particular combinations of auxotrophic markers in both types of strain, so that reciprocal crosses could be made which should

considerably facilitate the further characterization of this gene transfer system. Unfortunately no phenotype apart from that exhibited in a cross with a known 'recipient' has been found to distinguish 'donors' from 'recipients'. Although it is tempting to suspect plasmid involvement, extensive analysis of strains by agarose gel electrophoresis (Eckhardt, 1978) has failed to show a consistent plasmid difference between 'donor' and 'recipient' strains.

The most extensive genetic analysis of a phytopathogenic pseudomonad is that of Fulbright & Leary (1978) in *P. glycinea*. These authors have established linkage between nine independent phenotypes based on coinheritance frequencies, using the P class R factor R68 (Lacy & Leary, 1976).

It is a pleasure to record our thanks to Dr J. E. Crosse and Dr C. M. E. Garrett for their advice and encouragement during the course of this work and also for the provision of the wild-type strain. J.E. is supported by an S.R.C. CASE award (No. B1288) in collaboration with East Malling Research Station.

REFERENCES

- CLOWES, R. C. & HAYES, W. (editors) (1968). *Experiments in Microbial Genetics*, p. 184. Oxford: Blackwell Scientific Publications.
- CROSSE, J. E. & GARRETT, C. M. E. (1970). Pathogenicity of *Pseudomonas morsprunorum* in relation to host specificity. *Journal of General Microbiology* **62**, 315–327.
- ECKHARDT, T. (1978). A rapid method for the identification of plasmid DNA in bacteria. *Plasmid* **1**, 584–588.
- FARGIE, B. & HOLLOWAY, B. W. (1965). Absence of clustering of functionally related genes in *Pseudomonas aeruginosa*. *Genetical Research* **6**, 284–299.
- FREIGOUN, S. O. & CROSSE, J. E. (1975). Host relations and distribution of a physiological and pathological variant of *Pseudomonas morsprunorum*. *Annals of Applied Biology* **81**, 317–330.
- FULBRIGHT, D. W. & LEARY, J. V. (1978). Linkage analysis of *Pseudomonas glycinea*. *Journal of Bacteriology* **136**, 497–500.
- GARRETT, C. M. E., PANAGOPOULOS, C. G. & CROSSE, J. E. (1966). Comparison of plant pathogenic pseudomonads from fruit trees. *Journal of Applied Bacteriology* **29**, 342–356.
- GARRETT, C. M. E., PRUNIER, J.-P. & CROSSE, J. E. (1977). Bacterial canker of stone fruits: virulence of typical and variant forms of *Pseudomonas morsprunorum* in wound inoculations. *Report of East Malling Research Station for 1976*, 127–128.
- HOPWOOD, D. A. (1959). Linkage and the mechanism of recombination in *Streptomyces coelicolor*. *Annals of the New York Academy of Sciences* **81**, 887–898.
- LACY, G. H. & LEARY, J. V. (1976). Plasmid-mediated transmission of chromosomal genes in *Pseudomonas glycinea*. *Genetical Research* **27**, 363–368.
- TOWNER, K. J. & VIVIAN, A. (1976). RP4 fertility variants in *Acinetobacter calcoaceticus*. *Genetical Research* **28**, 301–306.