

Investigation of the mating system of *Pseudomonas aeruginosa* strain 1

III. Kinetic studies on the transfer of the sex factor (FP)

BY J. S. LOUITIT, M. G. MARINUS AND L. E. PEARCE*

Department of Microbiology, University of Otago, Dunedin, New Zealand

(Received 17 February 1968)

1. INTRODUCTION

Holloway & Jennings (1958) suggested that maleness in *Pseudomonas aeruginosa* was due to an infectious fertility factor which they called FP to distinguish it from the fertility factor (F) of *Escherichia coli*. Maleness is defined as the ability to transfer genetic material to another cell by cell-to-cell contact, and male strains (FP⁺) can transfer maleness as well as genetic material to a strain lacking the factor (FP⁻).

Very little is known about the sex factor of *P. aeruginosa* and there is certainly little information about its transfer during conjugation. In the original report, Holloway & Jennings (1958) showed that, when a recombinant donor (R629) was incubated overnight (shaken in broth) with a recipient culture of strain 1, twenty out of twenty-five re-isolated females had been converted to males. The only other information concerns the transfer of FP into recombinants. Holloway & Fargie (1960) reported variations in FP transfer from 0 to 71% and showed that the ability to transfer FP was a particular characteristic of the donor strains. In a strain 1 × strain 1 cross, eleven out of thirty-five recombinants (31%) were FP⁺. Cooke (1967) reported transfer into recombinants ranging from 3.3% to 96.6% depending on the recombinants selected.

These results were obtained from crosses carried out on plates with no restriction of pairing and do not provide information about the kinetics of transfer. If we are to understand the conjugational gene-transfer system of *P. aeruginosa*, such quantitative information must be obtained and considered, together with information about the kinetics of gene transfer (Loutit, Pearce & Marinus, 1968; Loutit & Marinus, 1968).

This paper is concerned, first, with the methods of testing for maleness and, secondly, with the kinetics of transfer of FP into recombinant and non-recombinant recipient cells.

2. MATERIALS AND METHODS

The media and methods of investigating the kinetics of transfer of genetic material have been described (Loutit *et al.* 1968). The derivatives of *P. aeruginosa*

* Present address: Dairy Research Institute, Palmerston North, New Zealand.

strain 1 used in the present work are listed in Table 1 together with their relevant characteristics.

The cultures to be tested for maleness were obtained as follows. Recombinant colonies were taken directly from the plates used in kinetic studies and patched (40/plate) on the same selective medium with sterile toothpicks. Non-recombinant recipient cells from the same mating were isolated as colonies by plating appropriate dilutions of the mating mixture on Brain Heart Agar (BHA) containing 1000 units of streptomycin/ml. After 2 days' incubation these colonies were patched on BHA.

Table 1. *Characteristics of strains of Pseudomonas aeruginosa used in the investigation*

Strain number	FP	str	Auxotrophic markers
OT1	+	<i>r</i>	<i>trp-1</i>
OT2	-	<i>s</i>	<i>leu-1</i>
OT8	+	<i>r</i>	<i>trp-1 ade-2</i>
OT9	-	<i>r</i>	<i>leu-1 lys-1</i>
OT10	-	<i>r</i>	<i>leu-1 ser-1</i>
OT15	+	<i>s</i>	—
OT47	-	<i>r</i>	<i>leu-1 ilvA12</i>
OT56	-	<i>s</i>	<i>leu-1 lys-1</i>
OT92	-	<i>r</i>	<i>ilvA12 his-2</i>
OT93	-	<i>r</i>	<i>his-2</i>
OT100	-	<i>r</i>	<i>leu-1 ilvA12 pro-4</i>

Symbols used for genetic markers: *ade* adenine; *his*, histidine; *ilv*, isoleucine and valine; *leu*, leucine; *lys*, lysine; *pro*, proline; *ser*, serine; *str*, streptomycin; *trp*, typtophan.

The recombinant and non-recombinant patched cultures, which can now be described as the suspected donor cultures, were tested for maleness by one of the methods below. A variety of recipients was used in the maleness testing but usually those with early markers were used because they yielded so many more recombinants. Any suspected donors which gave a positive result were restreaked on the same medium and retested using cultures obtained from single colonies.

(i) *Maleness testing from liquid culture*

The suspected donor colonies were inoculated into $\frac{1}{4}$ oz. Bijou bottles containing 2.5 ml. of nitrate Nutrient Broth (NB) and incubated overnight. The recipient cells were grown overnight in 10, 50 or 100 ml. volumes of nitrate Brain Heart Broth (BHB). This bulk culture was diluted with an equal volume of nitrate NB, 2.5 ml. volumes were pipetted into the suspected donor cultures and the mixtures incubated for 1-2 h. The bottles were shaken vigorously and one loopful was plated on the appropriate selective medium by spreading over an area 2-3 cm in diameter. The plates were then incubated for 2 days and examined for recombinants.

(ii) *Maleness testing by replica plating*

This is essentially the method described by Adelberg & Burns (1960). The suspected donor colonies were patched on fresh BHA (40 patches/plate) and incubated for 6 h. The recipient cells were grown overnight in nitrate BHB, an equal volume of medium was added and the culture was incubated for a further 3 h. The cells were then centrifuged and washed once in minimal medium. They were resuspended in one-tenth the original volume of minimal medium and 0.1 ml. (3×10^9 cells) was spread on the selective plates. The patched donor colonies were then transferred to sterile velvet and printed on the recipient lawn. The plates were incubated for 2-3 days and examined for recombinant colonies.

(iii) *Comparison of known male cultures*

This method relies on the assumption that there is a definite order of chromosome transfer in *P. aeruginosa*. If cells are mated for a fixed period of time under standard conditions the number of recombinants obtained for any marker would depend on the position of that marker in relation to the origin. The method is essentially the same as that described in (i). The main difference lies in the time of incubation, which was 4 h for these experiments but could be varied depending on the number of recombinants expected. After incubation of the plates the numbers of recombinants per loopful of mated cells were scored. It was not always possible to get an accurate count of the colonies but the main concern was to show gross differences and these were easily seen.

3. RESULTS

(i) *Maleness testing*

In preliminary tests for maleness, OT10 (*leu-1, ser-1*) was used as the recipient strain and various mating procedures were tried. A replica plating method was used and, in addition, drops of nutrient broth cultures of suspected males were spread on lawns of the female culture on minimal agar plates. Even with cultures of known males, it was not possible to get consistent results and frequently no recombinants were found. The problem was investigated and many factors were found to influence recombinant formation on the plate. The presence of traces of nutrient broth probably had the greatest single effect but its influence could be modified by other conditions such as the dryness of the plate and the amount of medium in the plates. The effect was particularly marked with genes transferred at low frequency and it was obvious that such methods would be unsuitable for studies on the kinetics of FP transfer.

Following the demonstration that consistent recombination frequencies could be obtained in the presence of potassium nitrate (Loutit *et al.* 1968), the method involving mating in liquid medium was developed. The technique had one great advantage in that it gave quantitative results and was adapted to compare the fertility of many different males. OT15 was selected in this way as the most active

culture from a large number of prototrophic recombinants isolated in a cross between OT8 and OT56.

Maleness testing with each test in a separate bottle was found to be very tedious and was quite unsuitable for kinetic studies, where hundreds of colonies had to be tested. Not only were large numbers of bottles involved but they had to be handled many times. The method was subsequently replaced by the described replica plating procedure but was retained to check the results obtained with the latter method.

The replica-plating method was developed after the demonstration that certain markers were capable of forming recombinants with relatively high frequency (Loutit *et al.* 1968). The *ilvA12* marker, which was the most efficient, was used for some experiments but showed a rather high reversion rate and also some cross-feeding and was eventually replaced by *his-2*. OT93, an *ilvA12*⁺ revertant of OT92, was used unless otherwise stated. This method was found to give results identical to those obtained with mating in liquid medium.

(ii) Kinetics of transfer of FP

(a) Unrestricted pairing

The kinetics of transfer of the gene *ilvA12* and the sex factor FP were studied in a cross between OT15 and OT47. Pairing was unrestricted, and to measure the recombinant levels dilution was made just prior to plating. To ensure that maximum figures were obtained for FP transfer, the samples were diluted 10⁻³ at sampling time and incubated for a further 30 minutes before plating on streptomycin BHA. This incubation ensured that all pairs capable of transferring FP would have done so. The results are shown in Table 2.

At 130 min, 59% of the recombinants and 23% of the recipient population had been converted to maleness. There appear to be two stages in the latter conversion. There was a period of 20 min producing 3-4% FP⁺ recipients and then a second period from 30 to 50 min raising this figure to 20%. Considering this high figure, it is interesting that the recombinants, constituting only 0.24% of the recipient population, were not all FP⁺.

(b) Restricted pairing

The transfer of FP was next investigated using the same donor and recipient strains but with pairing restricted at 20 min and diluting the cells 10⁻³ in nitrate NB. The *ilvA12*⁺ recombinants were scored and colonies of the recipient population were isolated at each sampling time without the additional incubation used in the previous experiment. The two classes of colonies were examined for maleness and the results are shown in Fig. 1.

The results are not very different from those obtained with unrestricted pairing (Table 2) but a number of points should be emphasized. The figures for restricted pairing are lower, as might be expected. The second period of transfer of FP to the recipient population was not apparent when pairing was restricted. There was little difference in the percentage of recombinants which were FP⁺ in the first 70 min. The relatively high figure shown at 20 min for restricted pairing is probably

Table 2. Transfer of the sex factor (FP) into the recipient population during unrestricted mating between OT15 and OT47

Time	Percentage of recipients FP+	Number of recombinants/ 10 ⁴ recipients	Percentage of recombinants FP+
0	0.8	0	—
10	4.2	0	—
20	3.3	2.3	21 (14)
30	3.3	3.3	30 (20)
50	18	14	26 (74)
70	16	31	34 (80)
90	19	68	37 (80)
110	19	110	43 (120)
130	23	242	58 (120)

120 recipients were tested for maleness at each sampling time. The numbers of recombinants tested for maleness are given in parentheses in the last column.

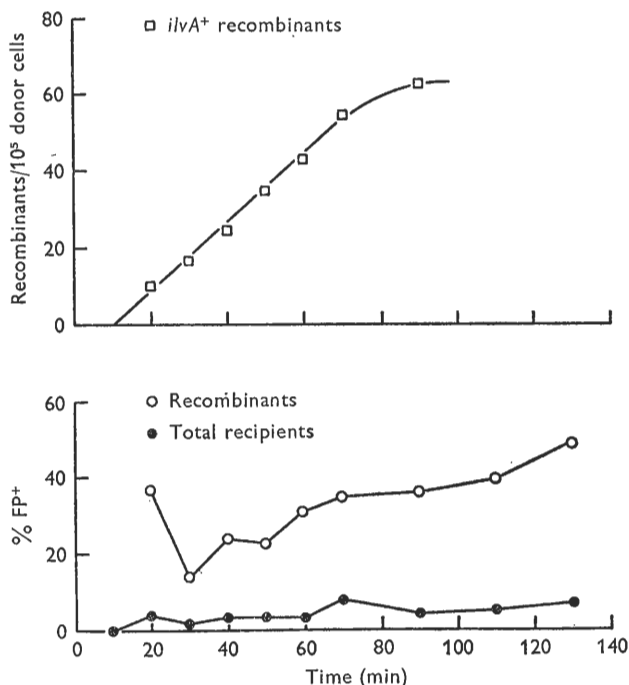


Fig. 1. Time of entry kinetics of the *ilvA12*⁺ marker (upper) and FP (lower) in a cross between OT15 and OT47 allowing 20 min for pairing. As well as the kinetics of FP transfer to the total recipient population, the percentage of the recombinants FP⁺ at each sampling time has been determined.

little significance, since the sample was very small (16). It is probable that a clear relationship exists between the percentage of recombinants which were FP⁺, and time. Comparison of the kinetics of transfer of genetic material and transfer

of FP to recombinants suggests that FP was already present in some of the recipients at the time of first appearance of the donor marker.

(iii) *Characteristics of freshly isolated males*

In considering the relationship between FP and chromosome, it is necessary to know whether freshly isolated males behave like OT15 and show the same oriented transfer of chromosome. Loutit *et al.* (1968) showed that OT15 transferred *ilvA12* with relatively high frequency (1 per 10^2 donor cells), *leu-1* with low frequency (1 per 10^7 donor cells) and *lys-1* with an intermediate frequency (1 per 10^5 donor cells). The method of comparing the different frequencies of transfer has been described and for the test itself duplicate cultures of 251 freshly isolated recombinant males were used with two recipient cultures (OT47 for *ilvA12*⁺ and *leu-1*⁺ recombinants and OT9 for *lys-1*⁺ and *leu-1*⁺). The number of recombinants of each class was scored and all the 251 males showed an obvious gradient of transfer. They gave almost confluent *ilvA12*⁺ colonies per loopful of mated cells, discrete numerous *lys-1*⁺ colonies and a few *leu-1*⁺ colonies. The 251 males were obtained using the original donor OT1 and not OT15, which is a highly selected strain.

As a further check, to ensure that the pattern in the recombinant males was not due to an association between FP and a particular segment of chromosome, a number of non-recombinant FP⁺ males was isolated from a cross between OT1 and OT2. Twenty-three were found following the examination of over 2000 cultures and all showed the same pattern of transfer as OT15. These results suggest that the relationship between FP and chromosome is a constant one in all these freshly isolated males.

(iv) *Linkage of ilvA12 and pro-4 to FP*

Kinetic studies showed that FP had already entered the recipients by the time of appearance of the first *ilvA12*⁺ recombinants. This raises the question as to whether the sex factor is linked to any of the early markers, although one would not expect this because of the low linkage figures (less than 40% for close markers). To test this, a cross was carried out between OT15 and OT100 (*ilvA12 pro-4 leu-1*), and the *ilvA12*⁺ and *pro-4*⁺ recombinants were isolated after 60 min. mating. Both classes were then examined for maleness so that the two known linkage groups (Loutit & Marinus, 1968) could be investigated. Of 74 *ilvA12*⁺ recombinants, 34 (43%) were males and of 80 *pro-4*⁺ recombinants, 27 (34%) were males. Obviously there was no linkage between the sex factor and the early markers since the *pro-4*⁺ class should show a much higher figure, being approximately five minutes closer to the origin.

4. DISCUSSION

Maleness testing of large numbers of cultures of *P. aeruginosa* strain 1 can now be carried out provided genetic markers, known to be transferred with relatively high frequency, are used in the tests. Since all freshly isolated males appear to have the same polarity of transfer only one or two recipient strains are required. As a consequence of the improved testing methods, the kinetics of transfer of FP in

