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

I. Kinetic studies

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1. INTRODUCTION

The conjugation system of *Pseudomonas aeruginosa* was first reported by Holloway (1955) but as yet it has not been fully investigated. The system was thought to be similar to the low-frequency mating system of *Escherichia coli* (Holloway & Fargie, 1960) but no high-frequency (Hfr) donors could be isolated. Analysis of low-frequency systems is always difficult and is further complicated by the variability exhibited by *P. aeruginosa*. Loutit & Pearce (1965*a*, *b*) showed that mating was a variable event susceptible to changes in environmental conditions since mating occurred on the selective plates following microcolony development.

Subsequently, Loutit & Marinus (1967) reported that, for certain markers, recombinants were formed with a frequency as high as 1 % and, furthermore, they were produced in liquid medium. The suggestion was therefore made that it might be possible to map part of the *P. aeruginosa* chromosome by determination of the the times of entry of certain markers into recipient cells.

This paper is concerned with the experiments which led to the preliminary report and includes further modifications which should allow more precision in subsequent mapping.

2. MATERIALS AND METHODS

(i) *Media*

The minimal medium was that of Davis & Mingioli (1950) and is referred to as liquid MIN medium or MIN agar. Amino acids as supplements were added at predetermined optimal concentrations ranging from 5×10^{-4} M to 2×10^{-3} M. Streptomycin was used at a final concentration of 1000 units/ml. The complete medium used was either Difco Nutrient Broth (NB) or Difco Brain Heart Infusion Broth (BHB). When solidified with agar, the latter is referred to as BHA. Certain media contained 0·4 % potassium nitrate and these have been referred to as nitrate NB or nitrate BHB.

Agar was added at 1·5 % for all solid media except that, for soft agar overlay, 0·6 % agar was added to MIN medium without glucose.

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(ii) *Organisms*

The original FP+ sub-lines of *P. aeruginosa* strain 1 from which all other strains have been derived were reported by Loutit & Pearce (1965*a*, *b*). These strains have now been renumbered as OT 1 and OT 2, respectively, and all derivatives have been numbered with an OT prefix in accordance with the recommendations of Demerec, Adelberg, Clark & Hartman (1966). Their numbers and genetic characteristics are shown in Table 1. Doubly auxotrophic mutants were obtained following ethyl methane sulphonate or *n*-methyl-*n*-nitroso-*N'*-nitroguanidine treatment and penicillin selection. Streptomycin-resistant mutants of the recipient strains were prepared by direct selection on BHA containing streptomycin.

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

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

	Characteristics		
Strain no.	FP	*str*	Additional markers
OT 1	+	*r*	*trp-1*
OT 2	−	*s*	*leu-1*
OT 8	+	*r*	*trp-1 ade-2*
OT 9	−	*r*	*leu-1 lys-1*
OT 10	−	*r*	*leu-1 ser-1*
OT 11	−	*r*	*leu-1 pro-1*
OT 12	−	*r*	*leu-1 met-2*
OT 15*	+	*s*	
OT 30	+	*r*	*trp-1 ilvA12*
OT 47†	−	*r*	*leu-1 ilvA12*
OT 56	−	*s*	*leu-1 lys-1*
OT 92	−	*r*	*his-2 ilvA12*
OT 101	−	*r*	*leu-1 his-6 ilvA12*

* OT 15 is a recombinant from a cross between OT 8 and OT 56.
† OT 47 is a recombinant from a cross between OT 30 and OT 11.

(iii) *Preparation of cells*

For all experiments incubation was at 37 °C. Recipient cells were used in the stationary phase after growth in nitrate BHB. Before use they were diluted 10^{-1} or 10^{-2} in nitrate NB to give approximately 3×10^8 or 3×10^7 cells/ml respectively. The donor cells were used in the logarithmic phase. The inoculum was obtained from a second subculture of the cells in nitrate BHB. From this culture sufficient cells were inoculated into 10 ml of nitrate NB to produce $3–5 \times 10^8$ cells/ml after $3\frac{1}{2}$ h incubation without shaking. It was found that shaking during incubation produced clumps which could not be dispersed easily. The log phase cultures were shaken vigorously just prior to use.

(iv) *Mating procedures*

The donor and recipient cell suspensions at either $3–5 \times 10^8$ or $3–5 \times 10^7$ cells/ml were mixed to give approximately equal proportions after a period of temperature equilibration. For some experiments pairing was unrestricted and samples (3–4 ml) were removed at intervals from the concentrated cells. Depending on the number of recombinants expected, the samples were plated without dilution or were diluted to a final concentration of $1·5–2·5 \times 10^5$ cells/ml and then plated. For other experiments pairing time was restricted to 5 or 20 min by dilution to a final concentration of $1·5–2·5 \times 10^5$/ml and samples were taken from these diluted cells.

Prior to plating 0·1 or 0·2 ml volumes in 3 ml of soft agar on selective MIN agar plates, samples or diluted samples (3–4 ml) were shaken on a Mickle shaker (H. Mickle, Gomshall, Surrey, England) for 1–2 min to interrupt mating. Streptomycin was included in the plates to contra-select the male. The plates were incubated for 2–3 days and scored for the presence of recombinants.

3. RESULTS

Preliminary investigations with various donors and the recipient OT 10 which carried the two markers *leu-1* and *ser-1* (90 % linked) showed that mating occurred in both MIN and complete liquid media. The frequency of mating was very low and recombinants appeared to be formed mainly at the surface pellicle. No recombinants appeared if mating was interrupted before 90 min.

The most important of these results was the demonstration of mating in the pellicle. It showed the difficulties of obtaining a reliable estimate of recombination frequency even in liquid medium. It also made possible an increase in the apparent efficiency of the system by mating under conditions where pellicle formation was at a maximum. In addition, an experiment was devised in which nitrate was included in the medium to determine whether recombination could take place in containers from which all air was excluded by filling them to the top and capping them. Recombinants were formed under these conditions provided the cells had been grown in nitrate broth, since the nitrate reductase is inducible. A comparison of two methods of mating showed a recombination frequency five times greater for cells mated in nitrate NB as opposed to mating in shallow layers of NB without nitrate.

Subsequent kinetic studies on the time of entry of different genes were carried out in the presence of nitrate using nitrate-induced cells. One other modification was included which increased the frequency of recombination by a further 30 %. This was the use of the second subculture in nitrate BHB instead of the first to provide the inoculum for the log phase donor cells.

(i) *Kinetic studies*

(*a*) *The* lys-1 *and* leu-1 *markers*

The early experiments were carried out with recipient strains having markers transferred with low frequency. Only one such experiment has been included. Donor strain OT 15 was mated with OT 9 (*lys-1 leu-1*). The mating mixture was not diluted and samples were removed at intervals and, after interruption of mating, plated to select for *lys-1*$^+$ and *leu-1*$^+$ recombinants. The results are shown in Fig. 1.

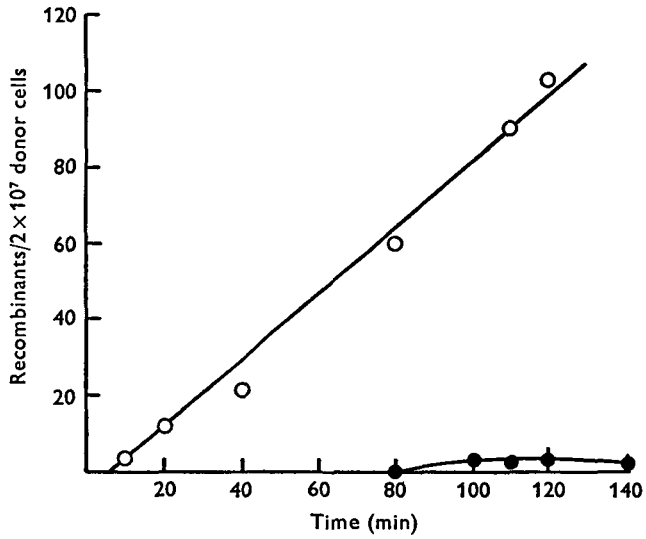

Fig. 1. Time of entry kinetics of *lys-1* and *leu*-1 markers (OT15 *str*^{s} × OT10 *lys-1 leu-1 str*^{r}). Pairing was unrestricted and cells were plated without dilution after mating had been interrupted on a Mickle shaker. ○, *lys-1*$^+$; ●, *leu-1*$^+$.

It can be seen that the *lys-1* marker entered early and with a greater frequency than *leu-1*, which appeared to enter at about 90 min. Unfortunately the number of *leu-1*$^+$ recombinants was not large enough to show a definite time of entry. When more cells were used to increase the number of recombinants the results were even more imprecise because low-level numbers were obtained throughout the experiment.

Other markers available at that time were also investigated but *lys-1* was certainly the most efficient. *Pro-1* (OT 11) and *met-2* (OT 12) entered somewhere between *lys-1* and *leu-1*. Thus some evidence of oriented transfer was obtained but generally the results for these markers were unsatisfactory.

(*b*) *The* ilvA *marker*

When experiments were initiated on the biosynthesis of isoleucine and valine in *P. aeruginosa* strain 1, it was immediately noticed that *ilvA* and *ilvB* were transferred with a very much higher frequency than any other marker previously examined. In the experiment recorded in Fig. 3, at 90 min there was one *ilvA12*$^+$

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recombinant for every 400 donor cells added at the start of the experiment (0·25 %). The same mating mixture left undiluted for 120 min gave approximately 1·2 % *ilvA12*$^+$ recombinants. The kinetics of transfer of *ilvA12* are shown in Fig. 2.

In this experiment, where pairing was a continuous process, any one sample contained many paired cells which had not completed gene transfer. To determine the total number of paired cells capable of gene transfer at each sampling time, pairing was restricted by dilution and the diluted cells were incubated for a further 40 min, a time previously shown to be sufficient for all pairs to complete gene transfer. Figure 2 shows that pairs continued to be formed for 20 min, after which the number of recombinants did not increase significantly for some time. Twenty minutes was selected as the pairing time for further kinetic studies.

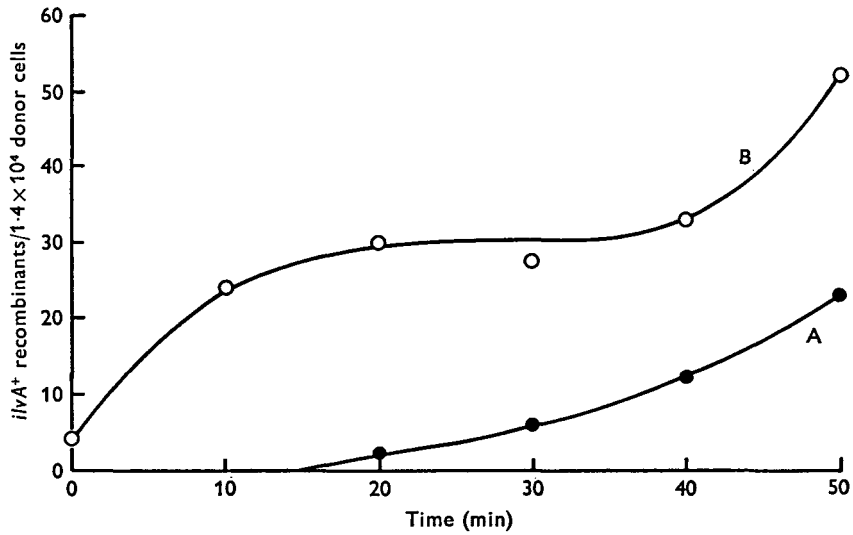

Fig. 2. A, The time of entry kinetics of the *ilvA12* marker (OT15 *str*^{s} × OT47 *ilvA12 leu-1 str*^{r}); pairing was unrestricted and the cells were diluted at sampling time, and mating interrupted, just prior to plating. B, Estimation of the total pairs capable of gene transfer at each sampling time; the same mating cells were used as in A and pairing was restricted at each sampling time by dilution in nitrate NB. The diluted cells were incubated for a further 40 min before interruption of mating and plating.

(*c*) *The* ilvA12, his-2 *and* his-6 *markers*

The recipient strain OT 92 was used for the first mapping experiments, and time of entry kinetics for *ilvA12* and *his-2* were established. OT 15 was used as donor strain and pairing was allowed to continue for 20 min before dilution. The results in Fig. 3 show a definite polarity of transfer and a measurable difference in the time of entry of the two markers. This suggests that the method can be used as a mapping procedure for such markers.

In a further experiment, OT 15 was crossed with another recipient OT 101 and the kinetics of entry of *ilva12* and *his-6* were determined. *His-6* was thought to be similar to *his-2* but the results in Fig. 4 show that *his-6* was transferred much earlier.

The results of two experiments are recorded in this figure and they were carried out simultaneously, using the same batches of donor and recipient cells. The difference in the two experiments was the time of pairing. In one, the cells were paired for

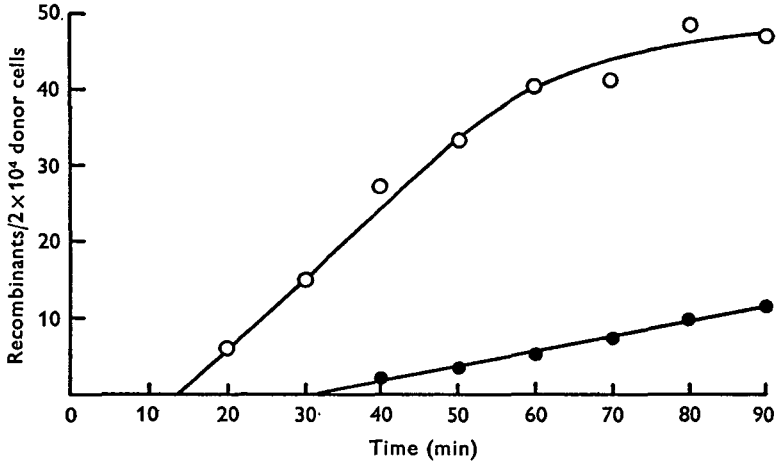

Fig. 3. Time of entry kinetics of *ilvA12* and *his-2* markers (OT15 *str*^{s} × OT92 *ilvA12 his-2 str*^{r}). The cells were diluted in nitrate NB after 20 min pairing. Samples for plating were removed from the diluted cells. ○, *ilvA12*; ●, *his-2*$^{+}$.

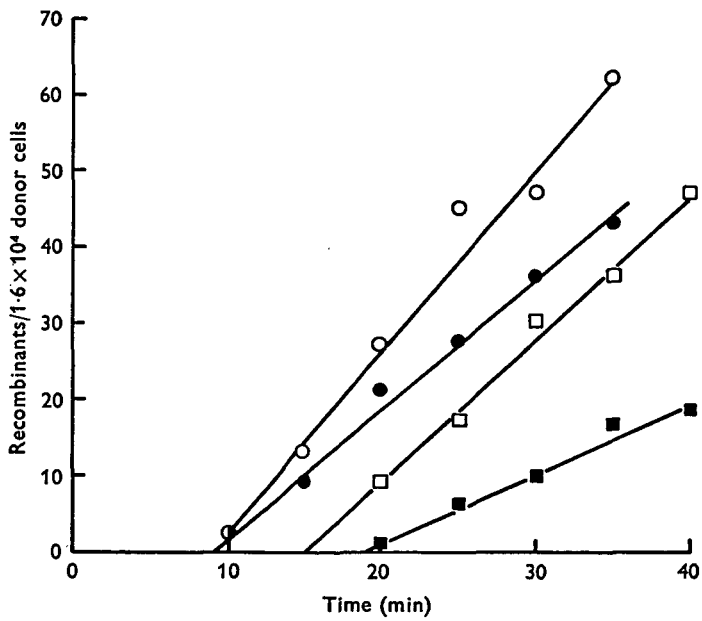

Fig. 4. The effect of dilution on the time of entry kinetics of *ilvA12* and *his-6* markers (OT15 *str*^{s} × OT101 *ilvA12 his-6 leu-1 str*^{r}). Pairing was restricted to 5 min when the cells were diluted in nitrate NB. Samples for plating were removed from these diluted cells as well as from the original undiluted material. This latter sample was diluted prior to plating. ○, *ilvA12*$^{+}$ diluted 10^{-3} at 5 min; ●, *ilvA12*$^{+}$ diluted at sampling; □, *his-6*$^{+}$ diluted 10^{-3} at 5 min; ■, *his-6*$^{+}$ diluted at sampling.

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5 min and then diluted, while in the other the cells were left to pair throughout the experiment and were diluted prior to plating. The 5 min pairing was used in an attempt to develop a method which would provide more precise information about the transfer of genetic material.

The two techniques gave different results. There was no difference in the time of entry of *ilvA12* but *his-6* entered 4 min earlier when pairing time was restricted. For both markers there was a significant reduction in the total number of recombinants where the cells were not diluted until the time of sampling. This difference disappeared if mating was allowed to continue and at 120 min there were many more recombinants with unrestricted pairing. The results with 20 min pairing were almost identical to those obtained with unrestricted pairing. Five-minute pairing, which gave the shortest interval between the markers, was adopted as standard for further kinetic studies.

DISCUSSION

In the investigation it became obvious that as soon as quantitative methods were employed, evidence of a polarity of transfer of genetic material was obtained. This was true even for markers transferred with low frequency. Only two of these markers are shown in Fig. 1: *lys-1*, which was certainly the most efficient of the low-frequency markers, and *leu-1*, the marker originally introduced by Holloway (1955), which turns out to be the most inefficient. A number of other markers were found to lie between these two extremes (Loutit, unpublished data).

In some ways it was unfortunate that much early work was carried out with recipient strains carrying this marker. On the other hand the transfer of *leu-1* was so much more susceptible to environmental conditions and the frequency of transfer was so low that it was possible and indeed essential to investigate the factors which influenced the transfer. The use of nitrate has been the most striking single factor influencing transfer and without it the interrupted mating techniques could not have been developed to such a degree of refinement. The other important factor influencing transfer was the use of a second subculture in nitrate BHB, which gave a 30 % increase in the number of recombinants.

The method finally adopted for estimating times of entry is very similar to that developed by de Haan & Gross (1962). It involves a 5 min pairing followed by a 10^{-3} dilution in fresh nitrate NB so that virtually no more pairs are formed. In fact this technique seems to be necessary to obtain the best information about the distances which separate the different markers. From the results shown in Fig. 4 it is obvious that where dilution at 5 min was used the apparent distance between the two markers was shortened. In a further experiment with the *his-2* marker, which was transferred very much later than *his-6* (cf. Figs. 3, 4), a difference of 7 min in the time of entry of *his-2* was observed with the two methods. Thus the apparent distances apart would be greater for the more distal markers. Dilution appears to trigger off some part of the transfer system and this is being investigated further.

It is interesting that a similar effect was observed for *Escherichia coli* K 12 by Loutit & Adelberg (1962, unpublished data) when it was shown that restriction of pairing time to 5 min markedly improved the time of entry of the *lac* marker in a cross between the Hfr strain AB 257 and an F^- recipient AB 266. Details of these strains can be found in a paper by Pittard, Loutit & Adelberg (1963).

There is little doubt that the method developed should allow a detailed investigation into the times of entry of certain genes, thus producing a map of part of the genetic material. There appears to be a definite polarity of transfer of markers, which naturally raises many questions about the relationship between the sex factor FP and the chromosome. Discussion of this requires information about the transfer of the sex factor and this will form the basis of a further communication.

SUMMARY

A method has been developed which will permit the mapping of the genetic material of *Pseudomonas aeruginosa*. This has been made possible by the use of potassium nitrate in the mating medium and other modifications which have increased the frequency of recombination. In certain circumstances, the *ilvA12* marker may be transferred by more than 1 % of the donor cells.

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