

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

II. Mapping of a number of early markers

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1. INTRODUCTION

The conjugational gene-transfer system of *Pseudomonas aeruginosa* has been used for mapping of chromosomal material and much valuable information has been obtained (Holloway, 1955; Holloway & Fargie, 1960; S. Howarth-Thompson & M. D. Cooke, personal communication; Cooke, 1967). The information is in the form of linkage data but it does not provide a clear picture of the whole chromosome or quantitative information about map distances.

Mapping of bacterial chromosomes by accumulation of linkage data is difficult and the best results have come from other methods (Wollman & Jacob, 1955, 1958) which use high-frequency (Hfr) donors. It is logical that, in the investigation of a bacterial species other than *Escherichia coli* K 12, attempts should be made to use similar methods, but unfortunately it has not been possible to isolate Hfr donor strains of *P. aeruginosa* (Holloway & Fargie, 1960; Loutit, unpublished data). Despite this, Loutit & Marinus (1967) and Loutit, Pearce & Marinus (1968) have shown that it should be possible to map certain genes by an estimation of their time of entry into suitable recipients. They showed a definite polarity of transfer of genes and that certain of these were transferred with a frequency sufficiently high to investigate the kinetics of gene entry.

This paper is concerned with the mapping of ten markers by a study of time of entry kinetics and a further study of linkages by unselected marker analysis.

2. MATERIALS AND METHODS

The media used and the methods developed to investigate the kinetics of genetic transfer have been described in a previous paper by Loutit, Pearce & Marinus (1968). Briefly, logarithmic-phase donor and stationary-phase recipient cell suspensions at $3-5 \times 10^8$ /ml in nitrate Nutrient Broth (NB) were mixed at 37 °C and left to pair for 5 min. They were then gently diluted 10^{-3} in fresh nitrate NB at 37 °C. Samples were removed at 5 min intervals, shaken on the Mickle shaker to separate the pairs and plated in soft agar on appropriate selective plates. In one experiment, to prevent double mating, the proportions of cells in the mating mixture were changed to four females for every male cell. After 5 min pairing the mixture was diluted so that there were 2×10^5 male cells/ml.

The characteristics and origins of the different strains discussed in the present paper are shown in Table 1. New mutations were induced as described by Loutit, Pearce & Marinus (1968) and *ilvA12*⁺ recipient strains were isolated as spontaneous revertants. New males were isolated during experiments on the kinetics of gene transfer and tested as described by Loutit, Marinus & Pearce (1968).

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

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

Strain no.	Characteristics				Additional marker	Origin
	<i>ilvA12</i>	<i>leu-1</i>	<i>str</i>	<i>FP</i>		
OT 1	+	+	<i>r</i>	+	<i>trp-1</i>	.
OT 15	+	+	<i>s</i>	+	.	.
OT 47	-	-	<i>r</i>	-	.	.
OT 94	-	-	<i>r</i>	-	<i>ser-5</i>	OT 47
OT 96	-	-	<i>r</i>	-	<i>arg-4</i>	OT 47
OT 97	+	+	<i>r</i>	+	<i>his-5 trp 1</i>	OT 1
OT 98*	-	+	<i>r</i>	-	<i>his-5</i>	OT 47
OT 100	-	-	<i>r</i>	-	<i>pro-4</i>	OT 47
OT 101	-	-	<i>r</i>	-	<i>his-6</i>	OT 47
OT 103	-	-	<i>r</i>	-	<i>phe-1</i>	OT 47
OT 104	-	-	<i>r</i>	-	<i>met-9</i>	OT 47
OT 105	-	-	<i>r</i>	-	<i>lys-2</i>	OT 47
OT 106	-	-	<i>r</i>	-	<i>his-7</i>	OT 47
OT 122	-	-	<i>r</i>	+	<i>phe-1</i>	OT 103
OT 124	-	-	<i>r</i>	+	<i>met-9</i>	OT 104
OT 183	+	-	<i>r</i>	-	<i>ser-5</i>	OT 94
OT 184	+	-	<i>r</i>	-	<i>his-6</i>	OT 101
OT 185	+	-	<i>r</i>	-	<i>phe-1</i>	OT 103
OT 186	+	-	<i>r</i>	-	<i>met-9</i>	OT 104
OT 187	+	-	<i>r</i>	-	<i>lys-2</i>	OT 105
OT 214	-	-	<i>r</i>	+	<i>his 6</i>	OT 101
OT 233	+	-	<i>r</i>	-	<i>arg-4</i>	OT 96
OT 234	+	-	<i>r</i>	-	<i>his-7</i>	OT 106
OT 235	-	-	<i>r</i>	+	<i>ser-5</i>	OT 94
OT 236	-	-	<i>r</i>	+	<i>pro-4</i>	OT 100
OT 237	+	-	<i>r</i>	-	<i>pro-4</i>	OT 100

* OT 98 was a recombinant forming a cross between OT 97 and OT 47.

Unselected marker analysis was carried out either on recombinants obtained during the kinetic studies or on others isolated following mating under the same conditions with recombinant selection by plating on the appropriate medium at 40 or 60 min. As a general rule, only the recombinants which had received the more distal markers were analysed for the proximal marker. The actual analysis was carried out by patching the particular colonies on appropriate agar plates with sterile tooth-picks. Usually they were patched first on to the medium, which would score the unselected marker, and then on to the control plate.

3. RESULTS

(i) Kinetic studies

Nine recipient strains of *P. aeruginosa* were used for these studies and the same donor OT 15 was used with each. The donor was prototrophic for each marker and the recipients carried the same *ilvA12* marker and one other known to be transferred within 30 min. In each experiment the time of entry of the new marker was compared with that of *ilvA12*. The results of the nine experiments are shown in Fig. 1.

The time of entry of the *ilvA12* marker was fairly constant between 8 and 10 min. The other markers also gave consistent times of entry and these ranged from 4–5

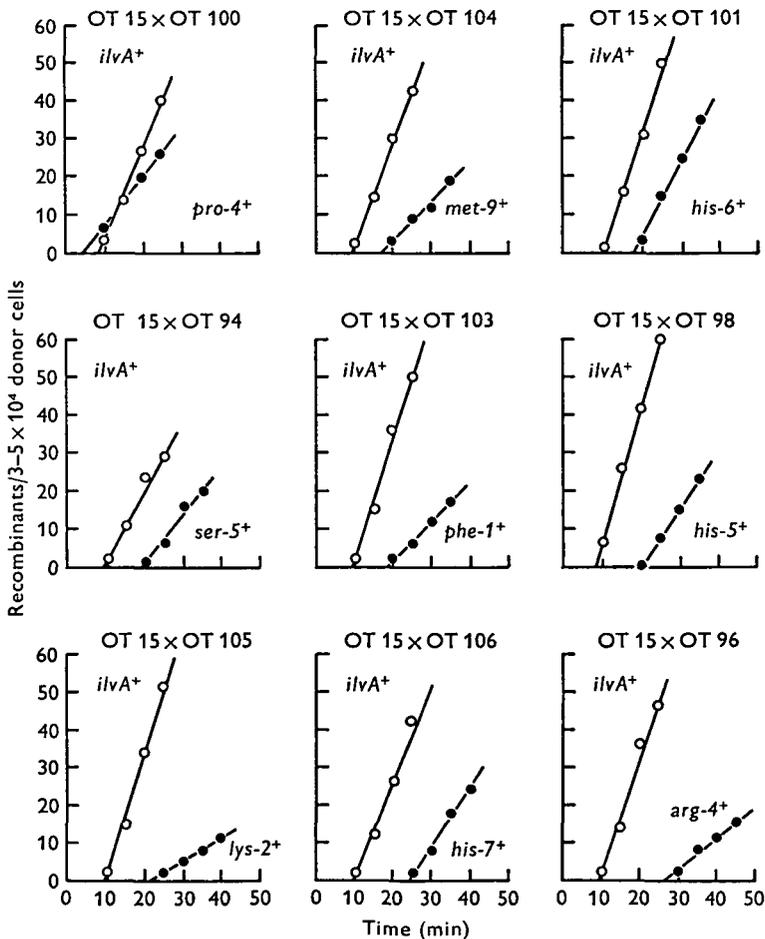


Fig. 1. Time of entry kinetics of ten genetic markers in nine recipient strains. Each strain carried the *ilvA12* mutation and the times of the others are referred to this. The donor was the prototrophic strain OT 15. The cells were paired for 5 min and then diluted in fresh medium. Samples were taken at intervals from the diluted cells and mating was interrupted before plating. Each point represents the mean of three plates.

min for *pro-4* to 27 min for *arg-4*. From this information a preliminary map (Fig. 2) of the ten markers can be drawn with estimates of their distances apart. The map can then be examined using unselected marker analysis to see whether it can be substantiated.

The method is not very precise and can only give an approximate idea of the position of the various markers. To get the exact order it would be necessary to have all of them in the same recipient.

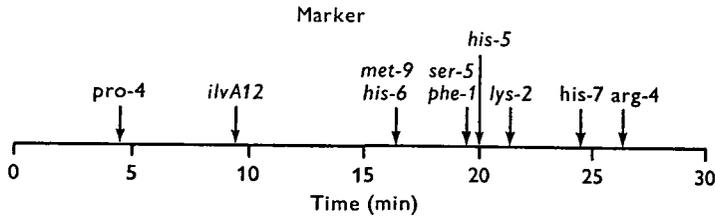


Fig. 2. Possible chromosome map based on the times of entry of ten markers in suitable recipients.

Table 2. Linkage of various markers to *ilvA12* measured as percentage of recombinants which have incorporated the two donor markers*

Cross	Selected marker	Total tested	Unselected marker	Double recombinants (%)
OT 15 × OT 100	<i>pro-4</i>	184	<i>ilvA12</i>	14
	<i>ilvA12</i>	184	<i>pro-4</i>	3
OT 15 × OT 104	<i>met-9</i>	184	<i>ilvA12</i>	2
OT 15 × OT 101	<i>his-6</i>	184	<i>ilvA12</i>	45
OT 15 × OT 94	<i>ser-5</i>	184	<i>ilvA12</i>	29
OT 15 × OT 103	<i>phe-1</i>	184	<i>ilvA12</i>	25
OT 15 × OT 98	<i>his-5</i>	80	<i>ilvA12</i>	16
OT 15 × OT 105	<i>lys-2</i>	184	<i>ilvA12</i>	13
OT 15 × OT 106	<i>his-7</i>	184	<i>ilvA12</i>	14
OT 15 × OT 96	<i>arg-4</i>	184	<i>ilvA12</i>	13

* With the exception of the first cross the most distal marker was selected and the proximal one was examined unselected.

(ii) Unselected marker analysis

In the time of entry studies the recombinants which had received the distal markers were examined for the presence of the donor *ilvA12*⁺ marker. The results, shown in Table 2, were quite unexpected. First, markers which on a time basis could be expected to be most closely linked to *ilvA12* were not as close as *his-6* and indeed the linkage figures of *met-9* and *his-6* to *ilvA12* were perhaps the most puzzling of all. These two markers appear to have entered the recipient cells at about the same time, but while *his-6* was 45% linked to *ilvA12*, *met-9* showed only 2% linkage. Secondly, the more distal markers or indeed all markers could have been expected to show much higher linkages to *ilvA12*. The results were so unusual that it was obvious that linkage figures were required for many other markers with-

in the set. Male strains carrying the various markers were prepared. They were streptomycin-resistant, so that streptomycin could not be used as a contra-selecting agent. To provide contra-selection the recipient strains were *ilvA12*⁺ revertants and all the selections were carried out on media without isoleucine, valine and streptomycin. The results of some of these crosses, arranged so as to show the order of the different markers, are shown in Table 3. The *pro-4* and *met-9* markers which gave anomalous results will be dealt with later.

Table 3. Linkage as measured by the percentage of recombinants which have incorporated two donor markers

Cross	Selected marker	Unselected marker	Double recombinants† (%)
OT 214 × OT 183	<i>ser-5</i>	<i>his-6</i>	94
OT 214 × OT 185	<i>phe-1</i>	<i>his-6</i>	46
OT 214 × OT 187	<i>lys-2</i>	<i>his-6</i>	12
OT 214 × OT 233	<i>arg-4</i>	<i>his-6</i>	10
OT 235 × OT 184*	<i>his-6</i>	<i>ser-5</i>	72
OT 235 × OT 185	<i>phe-1</i>	<i>ser-5</i>	59
OT 235 × OT 98	<i>his-5</i>	<i>ser-5</i>	49
OT 235 × OT 187	<i>lys-2</i>	<i>ser-5</i>	24
OT 235 × OT 234	<i>his-7</i>	<i>ser-5</i>	23
OT 235 × OT 233	<i>arg-4</i>	<i>ser-5</i>	19
OT 122 × OT 184*	<i>his-6</i>	<i>phe-1</i>	31
OT 122 × OT 183*	<i>ser-5</i>	<i>phe-1</i>	49
OT 122 × OT 98	<i>his-5</i>	<i>phe-1</i>	63
OT 122 × OT 187	<i>lys-2</i>	<i>phe-1</i>	30
OT 122 × OT 234	<i>his-7</i>	<i>phe-1</i>	34
OT 122 × OT 233	<i>arg-4</i>	<i>phe-1</i>	22
OT 97 × OT 94*	<i>ser-5</i>	<i>his-5</i>	30
OT 97 × OT 103*	<i>phe-1</i>	<i>his-5</i>	51
OT 97 × OT 105	<i>lys-2</i>	<i>his-5</i>	62

* Crosses marked in this way involve the isolation of proximal marker recombinants and the examination of the distal markers unselected. The results should be compared with those of the reciprocal cross elsewhere in the table.

† 104 colonies were investigated for each cross.

From this and the data in Table 1 it is possible to assign a definite order to eight of the markers which seem to fall on a single linkage group. They were linked but it is surprising how linkage diminished over apparently short distances of chromosome. The map of this particular linkage group is shown in Fig. 3, which is not drawn to scale and includes some linkage data. The relative positions of *lys-2* and *his-7* are not fixed unequivocally and the final order depended primarily on the times of entry of the two markers.

In drawing up this map two markers (*pro-4* and *met-9*) have been excluded, and these can be dealt with now. The first point to consider is their linkage to *ilvA12* and these results were included in Table 2. On the basis of their times of entry, *pro-4* and *met-9* were as close or closer to *ilvA12* than any of those already dealt with.

Despite this they showed very little linkage to *ilvA12*. Obviously their linkage to each other and to some of the other markers is of considerable interest. The recipient strain carrying the *met-9* marker was converted to maleness and also *ilvA12*⁺ revertants were isolated from it so that the linkage of *met-9* to the other three markers which entered recipients at approximately the same time (*his-6*, *ser-5* and *phe-1*) could be determined. A number of reciprocal crosses were made but none of the three genes was linked to *met-9*, which was a surprising result in view of the times of entry of these four markers.

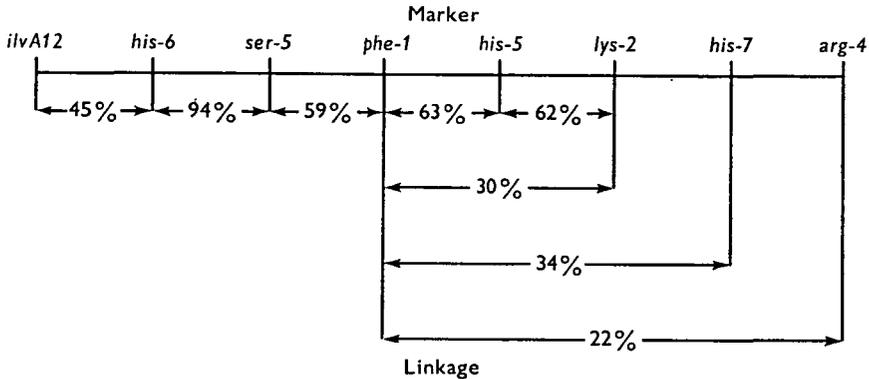


Fig. 3. Order of eight markers forming a single linkage group. The order is based on linkage analysis and times of entry in suitable recipients.

Table 4. Linkage of *pro-4* to *met-9* and *his-6* as measured by the percentage of *met*⁺ and *his*⁺ recombinants which have also incorporated *pro-4* donor marker

Cross	Selected marker	Unselected marker	Double recombinants (%)
OT 236 × OT 186	<i>met-9</i>	<i>pro-4</i>	25
OT 236 × OT 184	<i>his-6</i>	<i>pro-4</i>	2

The next possibility considered was that *pro-4* and *met-9* were linked to each other. A male strain carrying the *pro-4* marker (OT 236) was prepared by infecting OT 100 with FP and this strain carrying the proximal marker was used to determine the linkages. The results are shown in Table 4 and it seems clear that *pro-4* was linked to *met-9*.

One additional reciprocal cross was carried out between OT 124 and OT 237 as a further check on the linkage between *pro-4* and *met-9*. The *pro*⁺ recombinants were then examined for the presence of *met-9*, which was found in 8% of them. This compares with 25% for the reciprocal cross, thus confirming the direction of transfer because less linkage is expected when a proximal marker is selected and a distal one examined unselected.

There are thus two linkage groups and there is also evidence that the early marker on each group is occasionally transferred into a recipient from a single

donor. This can be deduced since the figures for *pro-4* and *ilvA12* in Table 2 were obtained from a mating mixture with a ratio of only one male for every four females.

4. DISCUSSION

The evidence in this paper confirms that it is possible to map at least part of the genetic material of *P. aeruginosa* by a study of the time of entry kinetics of certain early markers. The major point of interest, however, is the demonstration of two distinct linkage groups. Not only were the two groups transferred during the same mating but occasionally by the same donor to a single recipient. One of the groups carried eight genes transferred in an ordered way and showed linkage values which fit in with the idea of their being on one chromosome. Two other genes showed linkage to each other but not to the members of the first group.

The results can be interpreted in two ways. On the one hand they suggest that there were two chromosomes which occasionally were transferred together. Alternatively they suggest that there was a third linkage group in which *ilvA12* and *pro-4* were linked.

Discussion of these hypotheses must centre around the results of the cross between OT 15 and OT 100 presented in Table 2. In that experiment the *ilvA12*⁺ and *pro-4*⁺ recombinants were isolated after 60 min mating following 5 min pairing with the male as minority parent. At that time all pairs would have completed transfer, and the number of each class gives us some measure of the probability of the two genes being transferred singly. There were twice as many *ilvA12*⁺ as *pro-4*⁺ recombinants and, if we assume that these were the only class of recombinants formed, 67% were *ilvA12*⁺ and 33% were *pro-4*⁺. The probability that both would be transferred would then be 22% (67% × 33%), but this would be too high because other recombinants must surely be formed. The actual percentage of doubles was 6.6% and for this to be similar to the expected probability there would have to have been an additional 230 recombinants which were neither *ilvA12*⁺ nor *pro-4*⁺. This is rather a high figure and suggests that there was probably some exclusion if the results were due to the simultaneous transfer of two chromosomes.

The other hypothesis of a third linkage group cannot be supported by any positive evidence, but if it is correct we can at least determine the order of transfer from the unselected marker analysis in the same cross. Higher linkage values are obtained when we select for a distal marker and look at the proximal unselected, and the results in Table 2 are consistent with *pro-4* being distal to *ilvA12*.

At present we cannot distinguish between these two hypotheses. Linkage for many more markers will need to be examined and we will need to know something of the association of the sex factor and the genetic material before the questions can be answered.

SUMMARY

Ten markers have been investigated for their times of entry into auxotrophic mutants of *P. aeruginosa* strain 1. Using this information and unselected marker

analysis two linkage groups have been demonstrated. The results could be accounted for by assuming that there were two chromosomes or that a third linkage group was involved, but we cannot distinguish between these two hypotheses at the present time.

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