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The order of replication of chromosomal markers in *Pseudomonas aeruginosa* strain 1

I. Marker frequency analysis by transduction

BY R. J. BOOKER AND J. S. LOUTIT
Department of Microbiology, University of Otago, Dunedin, New Zealand

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SUMMARY

The generalized transducing phage F116 has been used to prepare lysates from fast- and slow-growing cultures of *Pseudomonas aeruginosa* strain 1. These lysates have been used to transduce a number of auxotrophic markers to prototrophy and the ratios of the numbers of transductants obtained with each lysate have been determined. Since the markers are those which have been mapped by conjugation in previous studies it has been possible to compare the ratios obtained for each marker with the relative position of the marker on the chromosome map. If the assumption is made that there is only one circular chromosome in *P. aeruginosa* strain 1 it is possible to suggest a way in which two apparently unlinked segments might be joined together. It is also possible to suggest that the chromosome replicates sequentially in two directions from a fixed origin.

1. INTRODUCTION

Interrupted mating techniques have permitted the mapping of large segments of the genetic material of *Pseudomonas aeruginosa* strain 1 (Loutit, 1969; Pemberton & Holloway, 1972). Although Pemberton & Holloway (1972) have decided that all their markers can be placed on one single chromosome, it has not yet been possible to show significant linkage between the two linkage groups described by Loutit (1969). The main difficulty with all these studies has been the inability to establish new donor strains with different transfer origins and until this happens the question is unlikely to be resolved genetically.

In view of these difficulties, it was decided to attempt to map a known set of genes (Loutit, 1969) using methods dependent on their order of replication rather than their order of transfer in conjugation. Of the various methods devised (Yoshikawa & Sueoka, 1963; Erickson & Braun, 1968; Cerdá-Olmedo, Hanawalt & Guerola, 1968; Masters, 1970; Masters & Broda, 1971; Caro & Berg, 1968; Wolfe, Pato, Ward & Glaser, 1968; Vielmetter, Messer & Schutte, 1968; Bird, Louarn, Martuscelli & Caro, 1972), that of Yoshikawa and Sueoka was chosen first since genetic transformation had been reported in *P. aeruginosa* strain 78 obtained from this laboratory (Khan & Sen, 1967). Since we could not repeat this work, however, the method of Masters (1970), which relied on transduction to measure gene frequencies, was selected. In this method, originally used with *Escherichia coli*, numbers of transductants from phage lysates obtained from fast-growing cells

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were compared with numbers obtained with lysates from slow-growing cells. A number of known gene markers was tested in this way. The ratios of the numbers from the 'fast-cell lysate' to the 'slow-cell lysate' varied from 2, for the genes near the site of initiation of replication, to 1 for those genes near the terminus. For genes between these two points the ratios of transductant frequencies were intermediate between two and one, and when the genes were arranged in the descending order of the ratios the order was the same as that obtained by conjugation mapping except that there was evidence of two-way replication (Masters & Broda, 1971).

The method has now been adapted to *P. aeruginosa* strain 1 and the results are recorded in this paper. There is evidence for two-way replication of the genetic material of *P. aeruginosa*, and if we make the assumption that the genetic material exists as a single closed-loop chromosome it is possible to suggest a way to join the two linkage groups of Loutit (1969). In any case it is possible to show that at least three of the four gene markers, and probably the fourth, on the smaller linkage group have very much smaller ratios than any of the others and thus can be assumed to be replicated much later.

2. MATERIALS AND METHODS

The original strains of *P. aeruginosa*, the methods of isolation of strains and the general media used have been described previously (Loutit, Pearce & Marinus, 1968; Loutit & Marinus, 1968). The strains used in the present study together with their relevant characteristics and derivations are listed in Table 1.

(i) *Bacteriophage.* The generalized transducing phage F116 was used in all experiments.

(ii) *Media.* The fast-growing cells and the various recipients were propagated in a minimal medium (Felkner & Wyss, 1970) supplemented with 1% BBL Yeast Extract, 0·4% Difco Brain Heart Infusion, 0·04% potassium nitrate and 0·5% glucose. The medium was further supplemented with the particular growth factors required by the strains. Generally they were provided at 10^{-4} M but isoleucine and valine were added at 10^{-3} M.

The slow-growing cells were propagated in the same minimal medium supplemented with 0·01% BBL Yeast Extract, 0·004% Brain Heart Infusion and 0·5% glucose. This medium was also supplemented with specific growth factors as described for the previous medium. For solid media, 2% agar was added.

The mean generation times of the fast- and slow-growing cells were 36 min and 75 min respectively.

(iii) *Preparation of lysates.* The strains were grown in 15 ml of the appropriate liquid medium in a shaking water bath at 37 °C until there were approximately 5×10^8 cells/ml. The adsorption of phage and the transfer of the infected cells was then carried out as quickly as possible and warmed glassware and media were used to prevent cold shock. The cells were deposited by centrifugation and resuspended in 1 ml of pre-warmed minimal medium supplemented with 10^{-3} M-$CaCl_2$. One ml

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Table 1. *Strains used with relevant characters and derivations*

Strain no.	Mutant genes	Derivation
OT 30	*ilvB trp-1*	Mutant from OT 1 (Loutit & Marinus, 1968)
OT 101	*ilvB his-6 leu-1*	Loutit & Marinus (1968)
OT 110	*ilvB trp-5 leu-1*	
OT 113	*ilvB met-11 leu-1*	
OT 119	*ilvB arg-6 leu-1*	
OT 124	*ilvB met-9 leu-1*	
OT 128	*ilvB ade-4 leu-1*	
OT 129	*ilvB pro-5 leu-1*	Loutit (1969)
OT 134	*ilvB met-12 leu-1*	FP^+ from OT 116 (Loutit & Marinus, 1968)
OT 236	*ilvB pro-4 leu-1*	FP^+ from OT 100 (Loutit & Marinus, 1968)
OT 262	*ilvB lys-2 leu-1*	FP^+ from OT 105 (Loutit & Marinus, 1968)

of phage suspension was added at a concentration which would provide one phage particle for ten cells and after shaking, the mixture was incubated at 37 °C for 10 min, centrifuged and the unadsorbed phage discarded. The deposit was re-suspended in 5 ml of fresh growth medium, 0·2 ml volumes were pipetted into 3 ml of soft agar (growth medium and 0·3% agar), which was then spread on pre-warmed plates of the same medium. For certain preparations as many as 50 plates were used. The plates were placed in plastic bags and incubated at 37 °C for 5 h for the 'fast' medium and 10–12 h for the 'slow' medium. The phage particles were then harvested as described previously (Pearce & Loutit, 1965; Marinus & Loutit, 1969).

(iv) *Transduction.* An overnight culture of the recipient in 10 ml nitrate Brain Heart Infusion was incubated for 2 h with an equal volume of the same medium. Just before the addition of the phage the cells were diluted 1:3 in the same medium (pre-warmed) and 2 ml of this suspension were added to an equal volume of the phage suspension at a multiplicity of 40 phage particles per cell. After 10 min at 37 °C, 0·2 ml volumes were pipetted into five soft agar tubes (1·5 ml volumes and 0·6% agar) each of which was then spread over a selective minimal agar plate. The plates were incubated at 37 °C for 3 days and scored for transductants.

For each recipient culture the following phage preparations were used: (*a*) phage propagated on a slow-growing donor; (*b*) phage propagated on a fast-growing donor; (*c*) phage propagated on a slow-growing recipient; (*d*) phage propagated on a fast-growing recipient.

3. RESULTS

(i) *Preparation of lysates.* The early attempts to obtain lysates from liquid culture as described by Masters (1970) were unsuccessful and a method was devised using solid media. Cells were grown first in liquid culture, infected and then transferred to solid media. The lysates prepared in this way contained sufficient phage particles to produce a reasonable number of transductants.

(ii) *Transduction.* The transductions were difficult for two reasons. We have found that strains prepared in this laboratory from original strain 1 cultures cannot be used for transduction if our strains are used as both donor and recipient and

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F 116 is the bacteriophage. Nevertheless, we have found that the numbers of transductants are sufficiently high if we use the original cultures (OT1 and OT2) as donor strains. In general, OT1 was used except where *trp-1* was the marker being studied. The other major difficulty was that we were concerned with known markers which had been mapped by conjugation. Some of these markers have relatively high reversion rates and when control plates were set up there were so many colonies that the numbers of transductants could not be scored. This was not a problem with conjugation where 1000-fold less cells were plated (Loutit *et al.* 1968). To overcome this problem we grew the phage on each recipient as well as the donor. These phage preparations were then used in exactly the same way as the transducing phage and the revertant colonies were scored on these plates. In this way we were able to include the killing effect of the phage and the numbers of revertants were reduced so that the transductants could be counted.

Table 2. *Means of ratios of transductants for lysates from fast- and slow-growing cells*

(Each ratio is compared with that obtained for *ilvB* which has been standardized as 1.)

Strain no.	Marker	Mean ratio	S.E.M.
OT 30	*trp-1*	2·36	0·24
OT 119	*arg-6*	2·14	0·19
OT 129	*pro-5*	2·11	0·23
OT 262	*lys-2*	2·11	0·27
OT 113	*met-11*	1·94	0·04
OT 110	*trp-5*	1·78	0·08
OT 236	*pro-4*	1·74	0·55
All strains	*leu-1*	1·57	0·06
OT 101	*his-6*	1·39	0·07
All strains	*ilvB*	1·0	—
OT 128	*ade-4*	0·67	0·14
OT 124	*met-9*	0·62	0·10
OT 134	*met-12*	0·55	0·13

Each recipient strain had the gene markers *ilvB112* and *leu-1* as well as one other. This meant that in almost every transduction, figures were obtained for all three markers using lysates obtained from fast and slow-growing donor cells. Ratios of transduction frequencies (comparing results with lysates from fast and slow cells for each marker) were then determined. As many as five experiments were carried out with each recipient and for the purposes of comparison the results in each experiment were adjusted so that the ratio of transduction frequencies for *ilvB112* was always one. Results are shown in Table 2.

The strains are arranged in the descending order of the ratios and at first glance little sense can be made of the order. When, however, the ratios are placed against the genetic map (Fig. 1) it can be seen that the two systems correlate reasonably well with *trp-1* showing the highest ratio and the markers on either side on the genetic map showing reduced ratios. This suggests that there is two-way replica-

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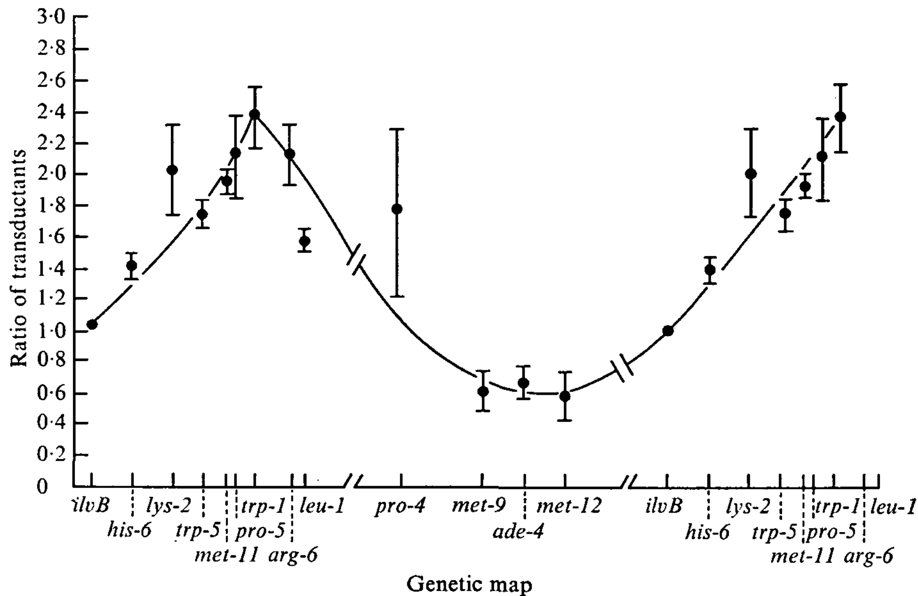

Fig. 1. Plot of means of ratios of transductants for lysates from fast- and slow-growing cultures against the genetic map.

Table 3. *Means of the ratios of transductants for lysates from fast-growing cells only*

(The ratio is the number of transductants for the marker compared with the number of *ilvB*$^{+}$ transductants.)

Strain no.	Marker	Mean ratio	S.E.M.
OT 129	*pro-5*	17·02	2·03
OT 113	*met-11*	8·24	0·84
OT 110	*trp-5*	6·29	0·62
OT 30	*trp-1*	6·27	0·92
OT 262	*lys-2*	4·90	0·97
OT 119	*arg-6*	1·90	0·17
All strains	*leu-1*	1·68	0·18
OT 101	*his-6*	1·00	0·09
All strains	*ilvB*	1·00	—
OT 128	*ade-4*	0·99	0·13
OT 236	*pro-4*	0·70	0·17
OT 124	*met-9*	0·22	0·24
OT 134	*met-12*	0·15	0·04

tion of the DNA of *P. aeruginosa* with the origin of replication somewhere near *trp-1*.

The ratios obtained for the four markers (*pro-4*, *met-9*, *ade-4*, *met-12*) on the second linkage group of Loutit (1969) are interesting because they also show a reasonable correlation between the ratios and the genetic map with three of them having ratios less than *ilvB* (1·0). This would suggest that they are replicated later than *ilvB*, either as the terminal region of the same replicating unit or as part of

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another chromosome. If they are on the same replicating unit then we think that the only way in which they can be fitted is that shown in Fig. 1. The main reason for this decision is that the ratio obtained for *pro-4* falls within the values obtained for the main linkage group and yet virtually no linkage can be demonstrated between *pro-4* and any of the markers in that group. For *pro-4* to be placed next to *ilvB* one would expect the ratio for *pro-4* to be less than one. It must be remembered, however, that the possibility that the four markers are in fact part of another chromosome cannot be ruled out by this technique.

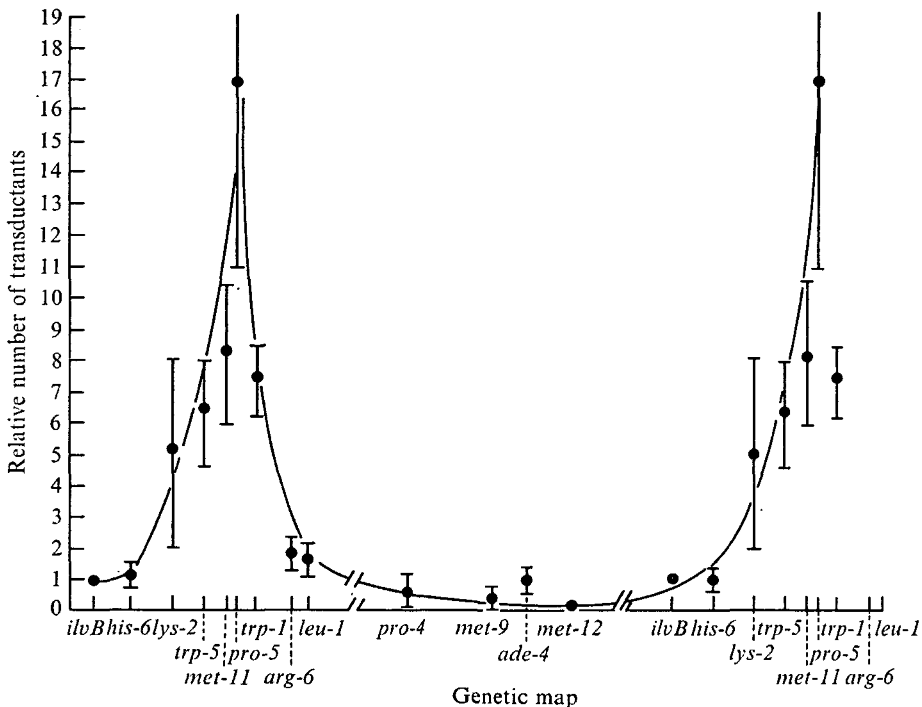

Fig. 2. Plot of means of transductants for lysates from fast-growing cultures only against the genetic map.

Masters & Broda (1971) suggested that for *E. coli* it was not necessary to compare fast- and slow-growing results and used the figures from the fast-growing-cell lysates alone. In Table 3 in this paper the transduction frequencies for each marker have been compared with the *ilvB*+ figures in the same experiment. The figures have been arranged in descending order and compared with the genetic map in Fig. 2. Again there is very good agreement although the origin of replication appears to have moved a little and the figure for *pro-4* is less than one.

The four markers of the small linkage group have been inserted in the same way as in Fig. 1 although they could just as easily be placed with *pro-4* next to *ilvB*. They have been left in the same relative position because the results obtained by comparing lysates from fast- and slow-growing cells should be more reliable. In

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any recombination there is always the possibility that certain genes will be incorporated more readily and this has been taken into account by comparing the results from two different lysates.

4. DISCUSSION

Even though Pemberton & Holloway (1972) have managed to include all their markers on a single linkage group it has not been possible to do the same in this laboratory. The experiments described in the present paper were initiated in the absence of any obvious method to solve the difficulty by conjugation or transduction mapping.

The first conclusion that can be drawn is that there appears to be two-way replication of the chromosome of *P. aeruginosa*, the origin of replication lying between 35 and 40 min on the genetic map (Loutit, 1969). *Pro-5* was mapped at 34 min and *trp-1* is very closely linked to *str-2* (unpublished data). Bi-directional replication has been reported in *E. coli* by Masters & Broda (1971) and in *Salmonella typhimurium* by Nishioka & Eisenstark (1970).

The second conclusion is that at least three of the four markers on the second linkage group of Loutit (1969) can be grouped together and separated from the major linkage group on the basis of their replication times which are later than any of the others. This supports the genetic evidence but does not determine whether the second linkage group forms part of the terminal region of a single chromosome or is part of a second independent replicating unit. Indeed this decision cannot yet be made although we are inclined to the former point of view. This conclusion requires the assumption, however, that the genome of *P. aeruginosa* is not greater than that of *E. coli*. If there were two chromosomes each about '50 min' in length and each replicating bi-directionally, we would not expect to find many genes replicating later than *ilvB*, which we know is about 25-30 min from the origin of replication. There is of course the other possibility that there are two chromosomes which replicate one after the other.

As stated above, we are inclined to the view that there is a single closed-loop chromosome for *P. aeruginosa* and have adopted this as a useful working hypothesis. On this basis it should be possible to join the two linkage groups and place the genes in their relative positions. We have attempted to do this in Fig. 1 based on the ratio obtained for *pro-4*, but it is interesting to see that without the control which takes the probability of integration into account, the position is not nearly so clear. The ratio obtained for *pro-4* is actually less than one and thus it would be possible to place it near *ilvB* rather than *leu-1*. It is interesting that the same pattern emerges if we plot the figures for the lysates from the slow-growing cultures and the *pro-4* figure is again less than that for *ilvB*. Nevertheless when we compare results for the lysates from the fast- and slow-growing cells the ratio for the *pro-4* marker is much higher than that for *ilvB* and this is the figure which should be accepted. The fact that the frequencies of *pro-4*$^+$ transductants are lower than the *ilvB*$^+$ for both lysates and yet the ratio (fast/slow) is much higher suggests that

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there may be a problem with the integration of the *pro-4* marker. At this stage there is some additional evidence (Booker & Loutit, in preparation) to support the contention that *pro-4* is replicated before *ilvB*, and *met-9*, *ade-4* and *met-12* some time after. In these experiments the back-mutation of the different genes was measured after treatment of synchronous cultures with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It is a modification of the method of Cerdá-Olmeda *et al.* (1968) and the results fit in well with those presented in this paper.

Thus it is proposed that there is a single closed-loop chromosome (Fig. 3) in *Pseudomonas aeruginosa* strain 1 and that it is likely to be longer than 70 min as measured in time-of-entry experiments. A necessary corollary to this is that there must be two sites of attachment for FP2 instead of the one suggested by Pemberton & Holloway (1972). This would be necessary to account for the relatively high-frequency transfer of both *pro-4* and *ilvB* in conjugation.

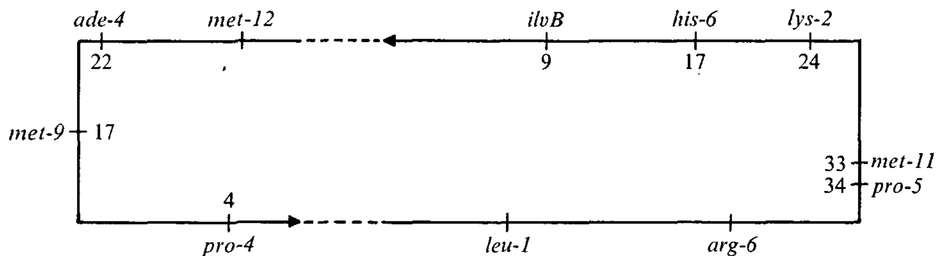

Fig. 3. Proposed orientation of the two linkage groups published by Loutit (1969). The numbers inside the figure indicate times of entry in minutes of the markers during conjugation and the arrows represent the sites of attachment of the sex factor FP2 and the direction of transfer.

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