Genet. Res., Camb. (1975), **25**, *pp.* 215–228
With 4 *text figures*
Printed in Great Britain

The replication of the chromosome of *Pseudomonas aeruginosa* strain 1

II. Sequential mutagenesis of synchronized cultures

By R. J. BOOKER and J. S. LOUTIT
Department of Microbiology, University of Otago, Dunedin, New Zealand

(*Received* 8 *July* 1974)

SUMMARY

The order of replication of a series of genes in *Pseudomonas aeruginosa* has been studied in synchronized cultures using a method based on the technique of sequential mutagenesis. This technique relies on the increased susceptibility of the replication point of the bacterial chromosome to mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The genes studied were those previously mapped by conjugation and whose order of replication had been studied by an investigation of gene frequencies in exponential populations. The results are consistent with the idea that there is two-way replication of the chromosome of *P. aeruginosa* starting at a point near *trp-1* and *arg-6*. They also confirm that the two linkage groups which have been found by conjugation replicate at different times. If the assumption is made that there is only one chromosome in *P. aeruginosa*, the results can be used to show how the two linkage groups may possibly be joined together and the order is such that there would have to be two sites of attachment for the sex factor FP2.

1. INTRODUCTION

On the basis of time of entry experiments and subsequent linkage analysis with strains of *Pseudomonas aeruginosa*, Loutit (1969) published a chromosome map consisting of two genetically unlinked groups of markers (Fig. 1).

The relationship between these two linkage groups is not clear and the work described in this paper and a preceding one (Booker & Loutit, 1974) was undertaken in an attempt to determine whether they are parts of a single chromosome or are separate physical structures.

In the preceding paper (Booker & Loutit, 1974) we reported the results of marker frequency analysis based on the ratios of transductants from phage lysates prepared from fast and slow-growing cells of *Pseudomonas aeruginosa* strain 1. The results showed that replication could be bidirectional from an origin near the *trp-1* marker and that at least three of the four markers on the smaller linkage group reported by Loutit (1969) were replicated later than the genes on the major linkage group. This information could not be used to provide evidence as to whether the linkage groups represent one or two chromosomes but it was used to

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

show how the two groups would have to be linked if they were joined into a single chromosome. The argument was based primarily on the fact that *pro-4* (the additional marker on the smaller linkage group) was replicated before the last marker of the major linkage group (*ilvB*). The results for *pro-4* were equivocal, however, and the present investigation was begun to confirm the earlier results and to see whether the suggested organisation of Booker & Loutit (1974) could be substantiated.

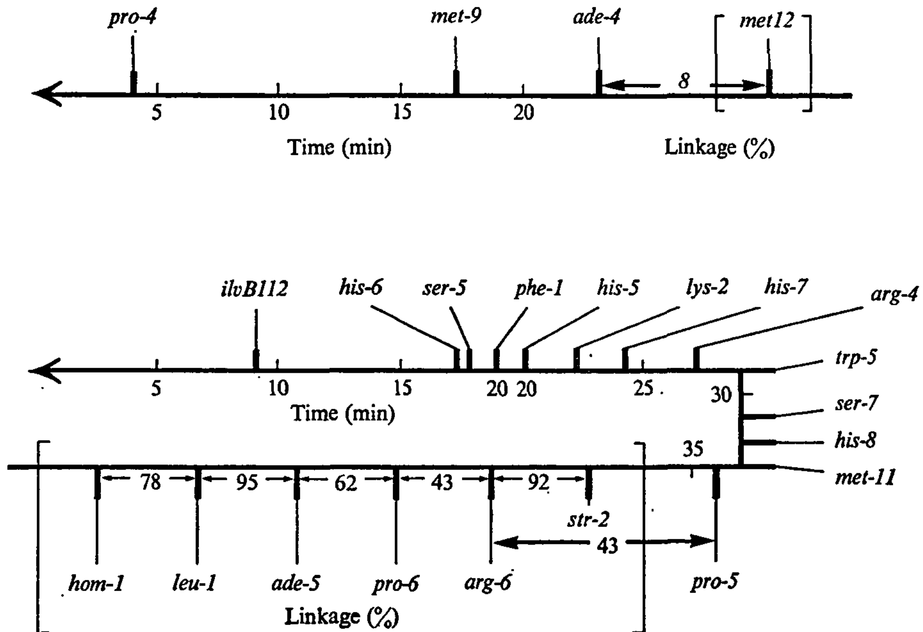

Fig. 1. The two linkage groups of *Pseudomonas aeruginosa* as described by Loutit (1969) indicating the relative positions of markers and the direction of transfer during conjugation.

In the present paper the order of replication was studied in synchronized cultures using a method based on the technique of sequential mutagenesis developed by Cerda-Olmedo, Hanawalt & Guerola (1968) and used since by Ward, Hane & Glaser (1970), Altenbern (1971) and Hohlfeld & Vielmetter (1973). This exploited the increased susceptibility of the replication point of the bacterial chromosome to mutagenesis by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NG). The mutagen treatment of successive samples from a synchronized culture and the scoring of treated cells for mutants resulted in a maximum number of mutants for particular genes occurring at the time of gene replication. The same strains were used as in the previous paper (Booker & Loutit, 1974) and the auxotrophic gene markers which had been mapped genetically (Loutit, 1969) were scored for reversion to prototrophy.

Two methods of synchronizing the populations of cells were employed. The first involved the aligning of the chromosome replication cycles by amino acid starvation

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

(Lark, Repko & Hoffman, 1963; Wolf, Pato, Ward & Glaser, 1968; Bird & Lark, 1968). This was followed by a period of protein synthesis in the presence of nalidixic acid, since protein synthesis is known to be necessary for initiation of chromosome replication in bacteria (Maaløe & Hanawalt, 1961; Lark *et al.* 1963; Lark, 1972) and nalidixic acid prevents DNA replication (Ward *et al.* 1970). Removal of the nalidixic acid resulted in synchronous re-initiation of chromosome replication. The second method was based on that of Altenbern (1971) and used phenethyl alcohol, which inhibits initiation of chromosome replication but permits completion of replication cycles already started. Thus, like amino acid starvation, phenethyl alcohol treatment results in alignment of chromosome replication in an exponentially growing population.

The results are consistent with our previous hypothesis that the chromosome of *P. aeruginosa* replicates bidirectionally from a point near the *trp-1* marker. They also support the contention that the *pro-4* marker is replicated before *ilvB* and that the other three markers on the second smaller linkage group (Loutit, 1969) are replicated after *ilvB*. In addition they provide evidence about the order of replication of the three distal markers on the smaller linkage group and allow a re-examination of the proposed orientation of the linkage groups if they represent two segments of a single chromosome (Booker & Loutit, 1974).

2. MATERIALS AND METHODS

(i) *Strains*

Most strains have been reported previously (Booker & Loutit, 1974) but all are recorded in Table 1 together with their relevant characteristics.

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

Strain no.	FP	Mutant genes	Derivation
OT30	+	*ilvB trp-1*	Mutant from OT1 (Loutit & Marinus, 1968)
OT100	−	*ilvB pro-4 leu-1*	Loutit & Marinus (1968)
OT101	−	*ilvB his-6 leu-1*	Loutit & Marinus (1968)
OT104	−	*ilvB met-9 leu-1*	Loutit & Marinus (1968)
OT109	−	*ilvB pro-5 leu-1*	Loutit & Marinus (1968)
OT110	−	*ilvB trp-5 leu-1*	Loutit & Marinus (1968)
OT113	−	*ilvB met-11 leu-1*	Loutit & Marinus (1968)
OT116	−	*ilvB met-12 leu-1*	Loutit & Marinus (1968)
OT119	−	*ilvB arg-6 leu-1*	Loutit & Marinus (1968)
OT124	+	*ilvB met-9 leu-1*	Loutit & Marinus (1968)
OT128	+	*ilvB ade-4 leu-1*	Loutit & Marinus (1968)
OT129	+	*ilvB pro-5 leu-1*	Loutit (1969)
OT132	+	*ilvB met-11 leu-1*	FP^+ from OT 113
OT134	+	*ilvB met-12 leu-1*	FP^+ from OT116
OT136	+	*ilvB arg-6 leu-1*	FP^+ from OT119
OT214	+	*ilvB his-6 leu-1*	FP^+ from OT101
OT236	+	*ilvB pro-4 leu-1*	FP^+ from OT100
OT262	+	*ilvB lys-2 leu-1*	FP^+ from OT105 (Loutit & Marinus, 1968)

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

(ii) *Media*

The basic growth medium was Difco Brain Heart Infusion supplemented with 0·4% potassium nitrate (Loutit, Pearce & Marinus, 1968). For synchrony by amino acid starvation and nalidixic acid treatment the following basal growth medium was used: 1 g ammonium sulphate, 2 g sodium citrate, 0·2 g magnesium sulphate, 0·3 g potassium dihydrogen phosphate, 0·7 g dipotassium hydrogen phosphate, 3 g Tris, 2·75 g maleic acid, 1·02 g sodium hydroxide, 1 l distilled water, pH 7·2. Growth factors were added as required at a concentration of 10^{-4} M except for isoleucine and valine which were used at 10^{-3} M.

(iii) *Establishment of synchrony following amino acid starvation*

All experiments were performed in a warm room with materials and equipment equilibrated to the temperature. Most experiments were performed at 30 °C but a few were carried out at 37 °C. Extensive preparatory experiments were performed in order to establish the conditions necessary to produce cell populations synchronized in chromosome replication.

(*a*) Choice of the amino acid to be removed during amino acid deprivation. An exponential phase culture at a cell density of approximately 5×10^8/ml was centrifuged and resuspended in separate flasks each containing 100 ml of fresh, pre-warmed medium lacking one or more of the amino acids required by all the strains used (isoleucine, valine and leucine). DNA synthesis was followed before and after restoration of the missing amino acids, which occurred 90 min later.

(*b*) Determination of the concentration of nalidixic acid necessary to inhibit DNA synthesis. An exponential phase culture was deprived of leucine for 90 min and then divided into separate flasks. Leucine and nalidixic acid at various concentrations were added to the flasks, and DNA synthesis was followed for a further 90 min.

On the basis of the results of these experiments a synchronization technique was formulated. The cultures were first grown overnight in nitrate Brain Heart Infusion; 0·1 ml of culture was then inoculated into 100 ml of supplemented growth medium in a 250 ml conical flask and this fresh culture was shaken overnight on an orbital shaker (180 rev/min). The following morning the contents, still in the exponential growth phase, were poured into 400 ml of fresh medium in a 1 l flask and shaken until the cells had increased 8-fold in number. They were harvested in a Sorval RC2-B superspeed centrifuge using a GSA rotor which was allowed to reach 10000 rev/min before being turned off. This would be equivalent to a sedimenting force of 16300g for 30 s. The cells were resuspended in 500 ml of growth medium lacking leucine and shaken for 90 min. Nalidixic acid was added to a final concentration of 20 μg/ml, 10 min later leucine was added and after 35 min shaking, the culture was centrifuged, washed in 500 ml of fresh fully supplemented medium and finally resuspended in the same volume of medium.

At the time of resuspension, and every 7·5 min subsequently for 90–120 min, two 10 ml samples were removed and centrifuged for 1·5 min at maximum speed

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

(i.e. 3200*g*) in a 'Doctor' centrifuge (Wifug, Sweden). Each pellet was resuspended and left for 15 min in 5 ml of Tris maleate buffer (Gomori, 1955) containing NG at a final concentration of 100 μg/ml. The cells were centrifuged, the pellets resuspended in 1·5 ml of unsupplemented growth medium and 0·1 ml volumes of appropriate dilutions were spread in 1·5 ml volumes of molten soft agar over two selective plates for each of two markers (one of which was always *ilvB*). Viable counts were made on each cell suspension and the revertants were scored on the selective plates after 3 days' incubation at 37 °C.

(iv) *Establishment of synchrony with phenethyl alcohol*

All experiments were performed in a warm room at 30 °C and materials and equipment were equilibrated to the temperature. The cultures were grown overnight in nitrate Brain Heart Infusion and 15 ml of culture were added to 250 ml of the same medium in a 500 ml conical flask and left for 4 h without shaking to provide about an 8-fold increase in cell number. Phenethyl alcohol was added dissolved in 10 ml of medium and the culture was left for a further 75 min to allow completion of chromosome replication. The final concentration of phenethyl alcohol was 0·1 % (v/v) for most strains and 0·2 % for OT236 and OT100. These concentrations were selected as being the minimum required to selectively inhibit the initiation of DNA synthesis, on the basis of results of previous experiments in which DNA synthesis was followed after the addition of various concentrations of phenethyl alcohol to a divided exponential culture growing in nitrate Brain Heart Infusion.

After treatment the culture was harvested in the Sorvall RC2-B centrifuge and resuspended in 300 ml of nitrate Brain Heart Infusion. Two 5 ml samples were removed at the time of resuspension and every 7·5 min subsequently and pipetted into tubes containing 0·1 ml of a solution of NG so that the final concentration was 100 μg/ml. After 15 min treatment with NG the cells were handled as described in (iii) above.

3. RESULTS

(i) *Synchrony following amino acid starvation*

The early experiments involving deprivation of different amino acids showed that only leucine starvation permitted both residual DNA synthesis and a rapid, linear increase in the DNA content of the culture following subsequent restoration of the amino acid. However, leucine starvation did not result in synchronous chromosome replication. It was for this reason that the nalidixic acid treatment was introduced and then it was possible to show synchronous replication of the DNA using the DNA estimation method of Giles & Myers (1965). It is interesting that the concentration of nalidixic acid necessary to inhibit DNA synthesis for short periods was very much less than that required for the inhibition of growth (500 μg/ml). Under these conditions in minimal medium at 37 °C, the mean generation time of the culture was about 68 min. The time required to complete replication of DNA after the removal of leucine was somewhat shorter than this, at about 50–60 min.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

Therefore, under conditions of synchrony, the graphs showing the DNA content of the cultures could be expected to show plateau regions between the first and second rounds of replication, but probably not beyond this (Cooper & Helmstetter, 1968). By varying the period of nalidixic acid treatment in the presence of leucine from 10 to 60 min and following DNA synthesis after resuspension in fresh medium, it was found that the most distinct plateau regions were obtained after 35 min nalidixic acid treatment, i.e. a total of 40 min with centrifugation time included.

In calculating the results the average number of revertants per fixed number of survivors was determined for each sample. It was important to count the survivors in each sample to avoid variations in the killing effect of the NG as well as losses

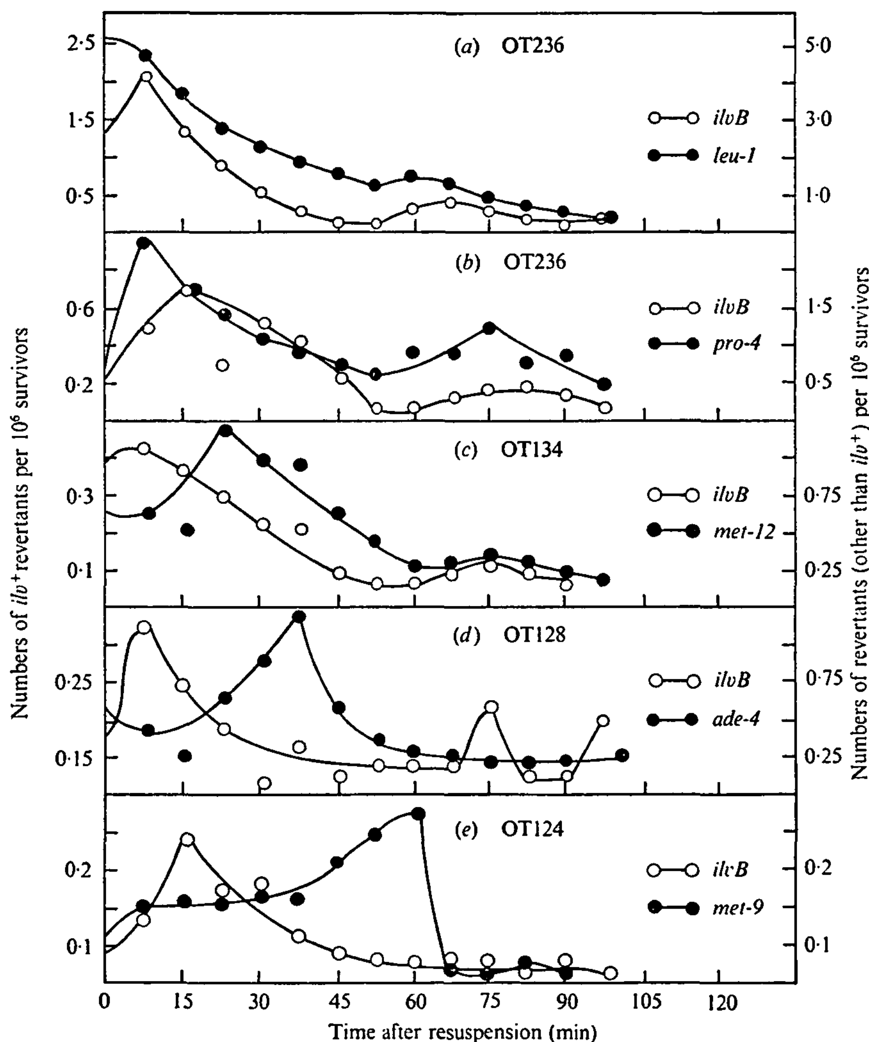

Fig. 2. Sequential mutagenesis of cultures after amino acid starvation and nalidixic acid treatment at 37 °C. Duplicate samples were treated every 7·5 min with 100 μg/ml NG for 15 min. Points represent duplicate samples with two replicates per sample.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

during centrifugation. The relative mean values for the duplicate samples taken at each sampling were then plotted against time and a graph was used to calculate the time of replication of each marker (the time at which the maximum number of revertants occurred). The absolute times obtained in repeat experiments were reasonably similar but, since *ilvB*$^+$ revertants were scored in each experiment and the time of replication of *ilvB* was estimated, all replication times have been recorded relative to *ilvB*.

The initial experiments were carried out at 37 °C using only FP$^+$ strains and the results are shown in Fig. 2. In graph (*a*) there is a peak of *leu-1* revertants at the time of the first sample, and another smaller peak about 60 min later, indicating that some degree of synchronous chromosome replication had continued into the second replication cycle. Two *ilvB* peaks are also evident about 7½ min later. A similar pattern is seen in graph (*b*) with the *pro-4* and *ilvB* peaks, although the second *ilvB* peak is less well defined. In graphs (*c*) and (*d*) the two *ilvB* peaks are 67½ min apart, closer to the 68 min expected from the mean generation time. It should be noted, however, that during the experiments it was not technically possible to sample at intervals of less than 7½ min and so these differences may not be significant.

Table 2. *Times of replication of genes relative to* ilvB *calculated from Fig. 2, as the differences between peak times for induced reversion during synchronous growth (37 °C) obtained by amino acid starvation and nalidixic acid treatment*

Strain	Marker	Time of replication
OT236	*leu-1*	7·5 min before *ilvB*
OT236	*pro-4*	7·5 min before *ilvB*
OT134	*met-12*	15 min after *ilvB*
OT128	*ade-4*	30 min after *ilvB*
OT124	*met-9*	45 min after *ilvB*

In Table 2 the replication time for each marker, selected as the time at which a peak number of revertants occurs, has been compared with that for *ilvB* in the same experiment. It can be seen, both from the graphs in Fig. 2 and from Table 2, that *leu-1* and *ilvB*, which are on opposite ends of the longer linkage group of Loutit (1969), replicated at approximately equal times, and that peaks for both these markers arose in the first few samples taken after resuspension of the nalidixic acid treated cultures in the fresh medium. No clear revertant peaks were obtained for the other markers on the longer linkage group of Loutit (1969). If the evidence of bidirectional replication obtained by transduction is correct (Booker & Loutit, 1974) then the results here suggest that the *leu-1* and *ilvB* markers are on opposite sides of the origin of replication, and that replication commences before the resuspension of the culture in the fresh medium. An examination of Fig. 2(*b*) supports this conclusion. In this graph the first *pro-4* peak is evident at 7½ min after resuspension, but the second peak occurred at 22½ min after the low point of the graph which signifies the end of one round of replication and the beginning of the next. It is therefore possible that replication commenced during the washing

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

procedure necessary to remove all the nalidixic acid from the treated culture. This washing step introduces a delay of at least 12 min between the removal of the nalidixic acid and the resuspension of the first sample in the nitrosoguanidine solution. Since this delay could not be reduced, it was decided to slow down chromosome replication by reducing the temperature. Subsequent experiments were thus performed at 30 °C when the Mean Generation Time was 108 min and the time of replication of the chromosome (*c*) was 90 min.

Table 3. *Times of replication of genes on the major genetic map relative to* ilvB *calculated as differences between peak times for induced reversion during synchronous growth (30 °C) obtained by amino acid starvation and nalidixic acid treatment*

Strain	FP	Marker	Relative time of replication
OT136	+	*arg-6*	45 min before *ilvB*
OT119	−	*arg-6*	45 min before *ilvB*
OT30	+	*trp-1*	45 min before *ilvB*
OT129	+	*pro-5*	37·5 min before *ilvB*
OT109	−	*pro-5*	45 min before *ilvB*
OT110	−	*trp-5*	37·5 min before *ilvB*
OT132	+	*met-11*	37·5 min before *ilvB*
OT113	−	*met-11*	37·5 min before *ilvB*
OT262	+	*lys-2*	22·5 min before *ilvB*
OT214	+	*his-6*	15 min before *ilvB*
OT101	−	*his-6*	15 min before *ilvB*
OT100	−	*pro-4*	15 min before *ilvB*
OT116	−	*met-12*	15 min after *ilvB*
OT108	−	*ade-4*	45 min after *ilvB*
OT104	−	*met-9*	52·5 min after *ilvB*

The replication times of the markers studied by sequential mutagenesis of cultures grown in minimal at 30 °C and synchronized by amino acid starvation and nalidixic acid treatment are listed in Table 3. The results obtained for some strategically placed markets are given in Fig. 3. It can be seen that the appearance of the *ilvB* peak was delayed at 30 °C, and in most cases occurred about 60 min after the time of resuspension of the synchronized culture in the fresh medium. The relative order of replication of the markers studied in the 37 °C experiments, and listed in Table 2, was found to be the same at 30 °C. The markers on the longer linkage group of Loutit (1969) were replicated before *ilvB*, supporting the conclusion that at 37 °C, chromosome replication was commencing before resuspension of the treated culture. There was no apparent difference for both FP^+ and FP^- strains, indicating that the presence of the sex factor has no influence on the replication of the chromosomes.

The results listed in Table 3 have been summarized in Fig. 4, where replication times of *ilvB*, have been plotted against the genetic map. This provides the evidence for bidirectional replication from a point somewhere near *trp-1* and *arg-6* and the relatively large difference in replication time between the linked markers *arg-6* and *leu-1* is interesting. There is also a large difference in the replication times of the linked markers *pro-4* and *met-9* on the smaller linkage group.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

(ii) *Synchrony following phenethyl alcohol treatment*

Sequential mutagenesis in broth was investigated as a possible means of producing more clearly defined peaks of revertants since it had been found that nitrosoguanidine treatment in broth yields a greater number of auxotrophs. Phenethyl alcohol was chosen as the most suitable means of synchronizing the cells in broth

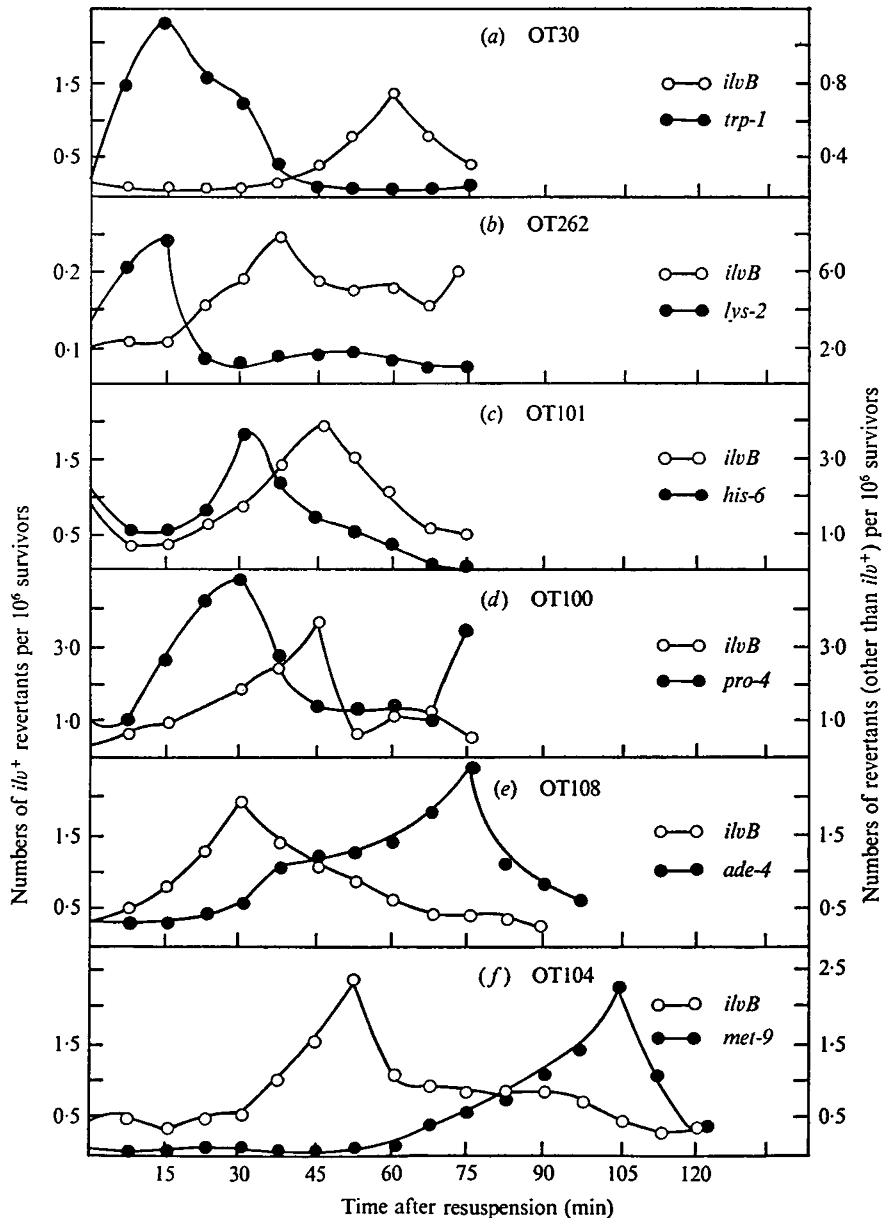

Fig. 3. Sequential mutagenesis of cultures after amino acid starvation and nalidixic acid treatment at 30 °C. Duplicate samples were treated every 7·5 min with 100 $\mu g/ml$ NG for 15 min. Points represent duplicate samples with two replicates per sample.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

and the experiments were performed at 30 °C in the hope of studying the replication times of the earlier replicated markers.

Although the results did produce more clearly defined peaks of revertants, it appears that chromosome replication in broth at 30 °C proceeded at a faster rate than in minimal at 30 °C since only the terminally replicating markers appeared. The replication sequence of those markers which were studied, however, was the same as before, with *leu-1* and *pro-4* replicating together and followed by *ilvB*, *met-12*, *ade-4* and *met-9*. The order was also the same for both FP+ and FP− strains.

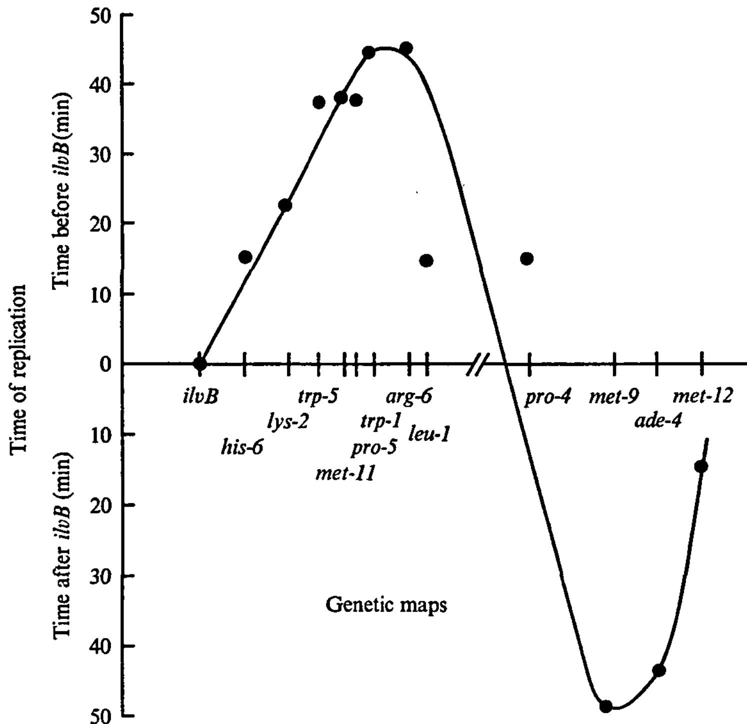

Fig. 4. Replication times of markers relative to *ilvB* determined by sequential mutagenesis of cultures synchronized at 30 °C by amino acid starvation and nalidixic acid treatment, have been plotted against the genetic map. Since no data were obtained for *leu-1* at 30 °C, the relative replication time for this marker has been calculated by multiplying the data at 37 °C by the ratio C_{30}/C_{37}.

4. DISCUSSION

In a previous paper (Booker & Loutit, 1974) the order of replication of the chromosome of *P. aeruginosa* strain 1 was determined by measuring the numbers of gene copies in an exponential culture and it was concluded that there was two-way replication starting at a point somewhere near *arg-6* and *trp-1*. In addition it was thought that *leu-1* and *pro-4* were replicated before *ilvB* and the three markers linked to *pro-4* on the smaller linkage group were shown to replicate some time after *ilvB*. The results were then used to show how the two linkage groups de-

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

scribed by Loutit (1969) could be joined together if it was assumed that *P. aeruginosa* had only a single closed-loop chromosome. It must be remembered, however, that this is only a working hypothesis and that there is as yet no physical evidence as to whether there are one or two chromosomes.

The present results are in agreement with those obtained by marker frequency analysis. The transduction ratios obtained in the marker frequency analysis experiments (Booker & Loutit, 1974) left some ambiguity when an attempt was made to show how the two linkage groups might be arranged if they are parts of a single chromosome. The present results, however, lend themselves much more easily to this. Sequential mutagenesis studies at 30 and 37 °C both show that *leu-1*, on the terminal end of the longer linkage group, and *pro-4* on the proximal end of the shorter linkage group, replicate at the same time. Both these markers replicate before the *ilvB* marker, which is on the opposite end of the longer linkage group from *leu-1*, and this indicates that the shorter group cannot be arranged with the *pro-4* and *ilvB* markers adjacent, as this would mean that the *pro-4* marker would replicate after *ilvB*. If these results are taken in conjunction with the genetic map of Loutit (1969) and are interpreted on the assumption that *P. aeruginosa* possesses a single circular chromosome (and there is some physical evidence to support this; Pemberton & Clark, personal communication) then a possible chromosome map emerges in which the origin of replication lies in the *arg-6* to *trp-1* region, and the terminus near *met-9*, with the two linkage groups arranged so that *leu-1* and *pro-4* are linked, as would be *ilvB* and *met-12*. A map incorporating these ideas has already been published (Booker & Loutit, 1974).

Cerda-Olmedo *et al.* (1968) were able to estimate the time taken for chromosome replication from the distances between consecutive peaks for the same marker in sequential mutagenesis studies, as they found that rounds of replication followed one another without a pause. In the present results, however, it appears that the time taken for chromosome replication is less than that required for cell division, so that a lag could be expected between consecutive rounds of replication. Although the exact time taken for residual DNA synthesis after the removal of leucine from the culture is difficult to determine using the diphenylamine method of estimating DNA, it is still possible to provide an estimate of the time taken for chromosome replication. In minimal medium at 37 °C the temperature at which the times of DNA transfer in conjugation have been determined (Loutit & Marinus, 1968) this time is 50–60 min. As yet, there is no available data on the relative rates of transfer and vegetative replication, but if the assumption is made that they may be similar, then a period of 50–60 min for vegetative chromosome replication proceeding bidirectionally may imply a total chromosome length of between 100 and 120 min. This figure is in good agreement with an estimate of 120 min obtained by Pemberton & Clark (personal communication). However, the experiments at 30 °C suggested that chromosome replication in broth is faster than in minimal, and since matings are performed in broth, this estimate of 120 min might by longer than is actually the case in conjugation studies, especially if the rates of transfer and vegetative replication are different.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

In conclusion we still do not know whether *P. aeruginosa* has one or two chromosomes. The early genetic evidence showed only that there were two distinct segments which could be transferred from any one donor population (Loutit & Marinus, 1968), suggesting three possible arrangements of the genes. There could be two chromosomes or there could be one with two ways in which transfer could be organized. For instance, there could be one site of attachment for the sex factor FP2 with chromosome transferred in either direction or alternatively there could be two sites of attachment with two different segments being transferred. We cannot yet decide about the number of chromosomes, but if we accept as a working hypothesis that *P. aeruginosa* has a single closed-loop chromosome, we can clearly distinguish between the other alternatives. For there to be a single site of attachment of FP2, with *pro-4* and *ilvB* both being transferred with relatively high frequency, *pro-4* and *ilvB* would have to be closely linked. However, the results of this paper and Booker & Loutit (1974) show that if the segments are joined it would not be possible for *pro-4* and *ilvB* to be closely linked. Thus there would have to be two sites of attachment for FP2 to produce the genetic results. Unfortunately it is not possible to prove this with our existing conjugating system and proof will have to await the development of a new system or the isolation of large numbers of mutants in the unknown regions which could then be investigated further by transduction.

This work was supported in part by a grant from the Medical Research Council of New Zealand.

REFERENCES

Altenbern, R. A. (1971). Marker frequency analysis mapping of the *Staphylococcus aureus* chromosome. *Canadian Journal of Microbiology* **17**, 903.

Bird, R. & Lark, K. G. (1968). Initiation and termination of DNA replication after amino acid starvation of *E. coli* 15T⁻. *Cold Spring Harbour Symposium on Quantitative Biology* **33**, 799.

Booker, R. J. & Loutit, J. S. (1974). The order of replication of chromosomal markers in *Pseudomonas aeruginosa* strain 1. I. Marker frequency analysis by transduction. *Genetical Research* **23**, 145.

Cerda-Olmedo, E., Hanawalt, P. C. & Guerola, M. (1968). Mutagenesis of the replication point by nitrosoguanidine: Map and pattern of replication of the *Escherichia coli* chromosome. *Journal of Molecular Biology* **33**, 705.

Cooper, S. & Helmstetter, C. E. (1968). Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology* **31**, 519.

Giles, K. W. & Myers, A. (1965). An improved diphenylamine method for the estimation of DNA. *Nature* **206**, 93.

Gomori, G. (1955). Preparation of buffers for use in enzyme studies. In *Methods in Enzymology* **1**, 138. New York: Academic Press.

Hohlfeld, R. & Vielmetter, W. (1973). Bidirectional growth of the *Escherichia coli* chromosome. *Nature, New Biology* **242**, 130.

Lark, K. G. (1972). Evidence for the direct involvement of RNA in the initiation of DNA replication in *Escherichia coli* 15T⁻. *Journal of Molecular Biology* **64**, 47.

Lark, K. G., Repko, T. & Hoffman, E. J. (1963). The effect of amino acid deprivation on subsequent DNA replication. *Biochimica et Biophysica Acta* **76**, 9.

Loutit, J. S. (1969). Investigation of the mating system of *Pseudomonas aeruginosa* strain 1. IV. Late markers. *Genetical Research* **13**, 91.

Loutit, J. S. & Marinus, M. G. (1968). Investigation of the mating system of *Pseudomonas aeruginosa* strain 1. II. Mapping of a number of early markets. *Genetical Research* **12**, 37.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press

LOUTIT, J. S., PEARCE, L. E. & MARINUS, M. G. (1968). Investigation of the mating system of *Pseudomonas aeruginosa* strain 1. I. Kinetic studies. *Genetical Research* **12**, 129.

MAALØE, O. & HANAWALT, P. C. (1961). Thymine deficiency and the normal DNA replication cycle. *Journal of Molecular Biology* **3**, 144.

WARD, C. B., HANE, M. W. & GLASER, D. A. (1970). Synchronous reinitiation of chromosome replication in *E. coli* B/r after nalidixic acid treatment. *Proceedings of the National Academy of Sciences, U.S.A.* **66**, 365.

WOLF, B., PATO, M. L., WARD, C. B. & GLASER, D. A. (1968). On the origin and direction of replication of the *E. coli* chromosome. *Cold Spring Harbour Symposium on Quantitative Biology* **33**, 575.

https://doi.org/10.1017/S0016672300015652 Published online by Cambridge University Press