Genet. Res., Camb. (1975), **25**, *pp.* 71–78
With 3 text-figures
Printed in Great Britain

Location of genes for the utilization of acetamide, histidine and proline on the chromosome of *Pseudomonas aeruginosa*

BY MARTIN DAY,* J. ROWLAND POTTS† AND PATRICIA H. CLARKE

Department of Biochemistry, University College London, Gower St, London WC1E 6BT

(*Received* 25 *November* 1974)

SUMMARY

Interrupted mating methods were used to map the positions on the chromosome of genes for the utilization of acetamide, histidine and proline. An entry time of 22 min was found for a *put* marker determining the utilization of proline. Two groups of *hut* mutants were studied. Mutation *hut-109* was located at 18 min and mutation *hutU108*, which results in a defect in urocanase activity, mapped at 28 min. *hutU* was shown by transduction with bacteriophage F116 to be linked to *ser-3* but not to *met-28* and *ilv-202*, giving a map order of *hutU–ser-3–ilv-202–met-28*.

The amidase genes were assigned an entry time of 65–75 min. A method is described for examining late entry markers by interrupted mating.

1. INTRODUCTION

A number of genes have been mapped on the chromosome of *Pseudomonas aeruginosa* strain PAO by interrupted mating combined with linkage analysis by transduction (Loutit & Marinus, 1968; Stanisich & Holloway, 1969; Pemberton & Holloway, 1972*a*). The data presented by Holloway and co-workers indicated that all the markers examined were carried on a single linkage group but Loutit & Marinus (1968) suggested that there were two separate linkage groups. However, a recent study by Booker & Loutit (1974) on the transduction frequencies of markers located at different sites has provided evidence to suggest that their two linkage groups may be parts of a single chromosome. Pemberton (1974), from data based on sedimentation analysis and electron microscopy, concluded that *P. aeruginosa* possesses a single chromosome of molecular mass $2{\cdot}1 \times 10^9$ daltons.

The recombination frequency for late markers in conjugal transfer by the sex factor FP2 was so low that Holloway, Krishnapillai & Stanisich (1971), Pemberton & Holloway (1972*a*) and Holloway (1975) were able to assign map positions only for markers entering in the first 50 min of mating. The markers mapped on the 0–50 min chromosome map region include genes for biosynthetic pathways, resistance to growth inhibitors and a few prophage attachment sites but no genes for

* Present address: Department of Biological Sciences, University of Warwick, Coventry, Warwickshire, CV4 7AL.

† Present address: Fermentation Development Department, Glaxo Laboratories Ltd, Ulverston, Cumbria.

catabolic enzymes. We have now devised methods for mapping catabolic genes and increased the sensitivity of the technique so that we have been able to map genes entering after the first 50 min of mating.

The amidase genes of *P. aeruginosa* strain PAC can be transferred by transduction using bacteriophage F116 isolated by Holloway, Egan & Monk (1960). The structural and regulator amidase genes were shown to be closely linked (Brammar, Clarke & Skinner, 1967) and transduction has been used for genetic fine structure analysis of mutants producing amidases with altered substrate specificities and with altered regulatory properties (Betz *et al.* 1974). The amidase phenotype of strain PAO is similar to that of strain PAC and interstrain transduction of genes for mutant amidases was carried out by Day & Clarke (1974). For mapping amidase genes on the chromosome we used PAO strains and these were also used for mapping the genes for histidine and proline utilization.

2. METHODS AND RESULTS

Strain PAO1 and some of the auxotrophic mutants were given to us by Professor B. W. Holloway. Acetamide-negative mutants were isolated from plates containing pyruvate-ammonium salts medium with fluoroacetamide by the method devised by Clarke & Tata (1973). Histidine-negative and proline-negative mutants were isolated by Mrs R. Tata as shadowy colonies on minimal agar plates, containing L-histidine or L-proline, respectively as the major carbon source for growth, supplemented with 0·002% sodium glutamate.

Transduction was carried out with bacteriophage F116 as described by Brammar *et al.* (1967). Method A used for interrupted mating was based on that described by Pemberton & Holloway (1972*a*) but their minimal salt medium used for plate mating, and for conjugal transfer in the interrupted mating experiments, contained glucose and citrate so that it could not be used for selecting catabolic markers. We replaced this with the minimal salt medium of Brammar & Clarke (1964) containing succinate as the sole carbon source to select for prototrophic recombinants and found that the numbers of recombinants obtained in plate mating with a range of known auxotrophic markers were comparable with those obtained using the methods of Holloway's laboratory. It was therefore possible to compare recombination frequencies for the catabolic markers with those reported previously for the auxotrophic markers. The minimal agar plates used to select recombinants contained succinate for auxotrophic markers and the appropriate carbon source for the catabolic markers. The conjugal mixture was incubated in the basal salt medium in the absence of a nitrogen or carbon source. Method A was used to map the *hut* and *put* mutations.

A preliminary estimate of the probable map position of the amidase genes was obtained by extrapolation from published data combined with plate mating experiments with some auxotrophic and amidase mutants. From data presented by Stanisich & Holloway (1969) it could be seen that the recombination frequency in plate mating was much lower for the later markers and this effect was

particularly clear-cut with the increased fertility mutant isolated by Pemberton & Holloway (1972*b*). The usual method of interrupting mating did not separate the mating pairs when this mutant PTO355 was used as the donor. Using the data of Pemberton & Holloway (1972*b*) we concluded that the numbers of recombinants declined exponentially with the distance of the markers from the origin of transfer, and obtained a linear relationship by plotting the log of numbers of recombinants against the time of entry for known markers. We then carried out standard plate

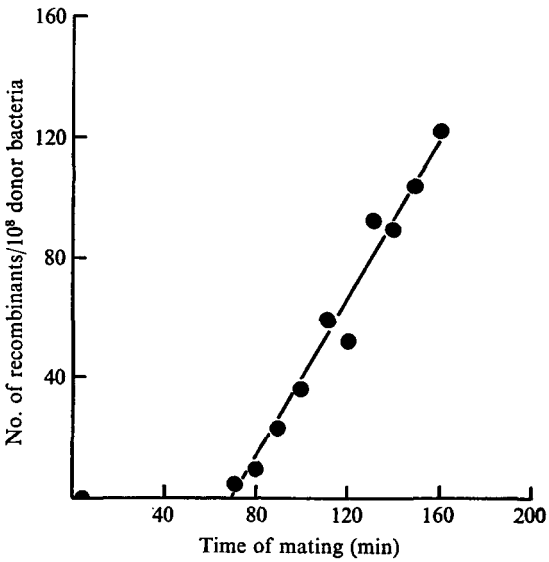

Fig. 1

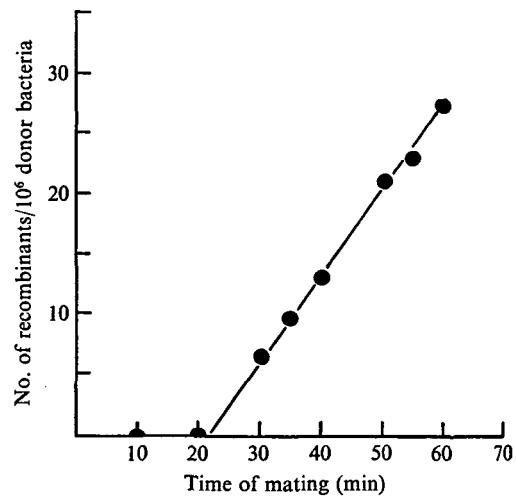

Fig. 2

Fig. 1. Interrupted mating Method B. Donor strain PAO381 FP2+, recipient strain PAO1632 FP2− *ami-151*. Time of entry of amidase genes 65–75 min. Selection on acetamide minimal agar plates.

Fig. 2. Interrupted mating Method A. Donor strain PAO381 FP2+, recipient strain PAO1635 FP2− *put-101*. Time of entry of *put* gene 22 min. Selection on proline minimal agar plates.

matings with strain PAO381 FP2+ as donor and auxotrophic and amidase-negative mutants as recipients. The number of recombinants obtained for an auxotrophic marker of known entry time was adjusted to be equivalent to that for a marker of about the same entry time when strain PTO355 had been used as donor. This was necessary since strain PTO355 gives higher numbers of recombinants than PAO381 (Pemberton & Holloway, 1972*b*). The numbers of recombinants for the amidase marker using strain PAO1632 as recipient were scaled up in the same way and this indicated a minimum time of entry > 55 min. By using a series of known markers with the same donor it should be possible to develop this method to give a preliminary estimate of the map position of any unknown genetic marker. We used it later to get a preliminary estimate of the map position of *hut* and *put* markers.

The low numbers of recombinants obtained in plate mating, and the indication

from the preliminary experiments that the amidase genes had a very late entry time, meant that the sensitivity of the interrupted mating method had to be increased. The interrupted mating method of Pemberton & Holloway (1972*a*) was first modified by using a volume of 10 ml instead of 25 ml in the mating mixture and plating out 0·4 ml instead of 0·2 ml in the agar-layered plate. This effectively increased the concentration 5-fold and made it possible to recover acetamide-positive recombinants. The estimated entry time was before 100 min and probably around 80 min. The numbers of recombinants recovered were too low to give a more exact value. Method B, which was finally adopted, was as follows. The donor and recipient cultures were grown as described by Pemberton & Holloway (1972*a*) but

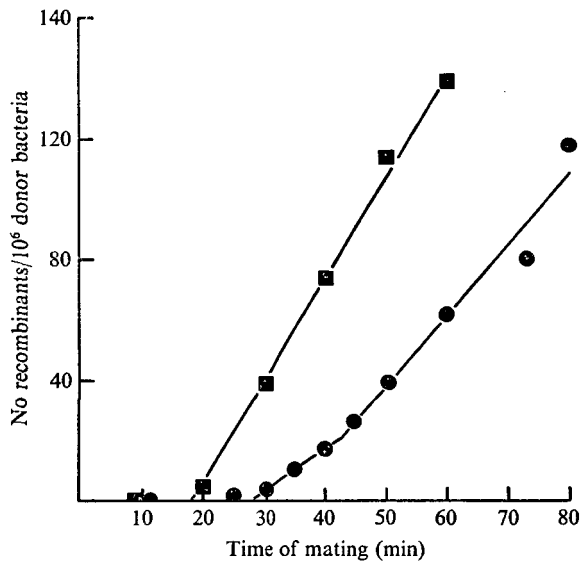

Fig. 3. Interrupted mating Method A. Donor strain PAO381 FP2+. ●, Recipient strain PAO1632 FP2− *hutU108*; time of entry of *hutU* 28 min. ■, Recipient strain PAO1879 FP2− *hut-109*; time of entry of *hut* gene 18 min. Selection on histidine minimal agar plates.

the conjugal mixture contained 2 ml donor culture PAO381 FP2+ (log phase, 10^9 cells/ml) and 4 ml recipient culture PAO1632 FP2− (stationary phase, 2×10^9 cells/ml). The conjugal mixture was divided into 0·5 ml portions and incubated in a water bath at 37 °C. At intervals mating was stopped by the addition of 0·5 ml of a preparation of phage E79 and the tubes were agitated to separate the mating pairs. Samples of 0·5 ml were added to 2·5 ml overlay agar and plated on acetamide plates. The frequency of revertants from donor and recipient cultures was $< 10^{-9}$.

Fig. 1 gives the results obtained for the amidase marker with method B. The time of entry of the amidase genes was estimated as 65–75 min. It was observed that the number of recombinants on the plates prepared from samples taken after 180 min of mating was about 4-fold greater than that found with the standard plate mating method using the same donor and recipient cultures. A procedure of incubating the

mating mixture in minimal medium for 2–3 h before plating on selective media might be useful for preliminary studies on markers which give low recombination frequencies in the standard plate mating method and are suspected to have late entry times.

Table 1. *Strains of* Pseudomonas aeruginosa

Strain*	Parent	Relevant genotype	Reference
PAO2	PAO1	*ser-3*	Pemberton & Holloway (1972 *a*)
PAO8	PAO1	*met-28*, *ilv-202*	
PAO381	PAO38	*leu-38*, FP2+	
PAO1632	PAO1870	*ami-151*, *hutC107*, *hutU108*	This paper
PAO1879	PAO1870	*ami-151*, *hutC107*, *hut-109*	
PAO1635	PAO1632	*ami-151*, *hutC107*, *hutU108*, *put-101*	
PAO1636	PAO1632	*ami-151*, *hutC107*, *hutU108*, *put-102*	
PAO1637	PAO1632	*ami-151*, *hutC107*, *hutU108*, *put-103*	
PAO1870	PAO1	*ami-151*, *hutC107*	

* Mutants were isolated after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG) at each successive step. Phage-resistant mutants (resistant to phage E79) were isolated by spreading 0·1 ml of a broth culture (5×10^9 cells/ml) on a nutrient agar plate and spotting a few drops of a preparation of phage E79 on the surface. Each mutant strain used as a recipient was made resistant to phage E79 independently.

The acetamide-negative mutant carrying *ami-151* was a spontaneous mutant isolated from fluoroacetamide-pyruvate medium. This was used as the parent for the isolation of the *hut* and *put* mutants (Table 1). Strains PAO1634 and PAO1636, both of which were *ami*$^-$ *hut*$^-$ *put*$^-$, were used as recipients in plate mating with strain PAO381 as donor. From the numbers of recombinants obtained it was estimated that the *hutU* mutation was likely to be in the 25–35 min region of the chromosome and the *put* mutations in the 15–25 min region. Analysis of the exconjugants from these crosses showed no linkage between *ami* and either *hutU* or *put*. Selecting for *put* in the cross PAO381 × PAO1635 gave 3% linkage for the non-selected marker *hutU* (3/98) and for the cross PAO381 × PAO1636 a linkage of 5% (2/38). Selecting for *hutU* gave no linkage with the non-selected *put* marker in either cross (0/50 in both experiments).

An interrupted mating experiment with PAO381 as donor and PAO1635 as recipient gave a time of entry of 22 min (Fig. 2) for the *put* marker. The other *put* mutants were not examined in detail but they were similar in phenotype and the numbers of recombinants obtained in plate mating with PAO1635 and PAO1636 would suggest that they were probably at the same gene locus. The *ami*$^-$, *hut*$^-$, *put*$^-$ mutants were used as recipients in transductional crosses with the donor lysate prepared on strain PAO1. Transductants were recovered for all three markers but there was no cotransduction of *hutU* with any of the *put* markers. It was also shown that there was no cotransduction of the *put* markers of strains PAO1635 and 1636 and the *hut* mutation of strain PAO1634 which was known to be at a different site from the *hutU* mutation (Potts, 1975).

Strain PAO1632 is a urocanase-negative mutant derived from a strain constitutive for the synthesis of histidine degradative enzymes (Table 1). Interrupted

mating with this strain as recipient, and strain PAO381 as donor, gave an entry time for the *hutU* marker of 28 min (Fig. 3). This is a region of the chromosome which has been carefully mapped by Holloway and co-workers and it was possible to test for linkage to known auxotrophic markers. Transduction with PAO1632 as donor and PAO2 as recipient gave values for cotransduction indicating linkage between *ser-3* and *hutU108* but with strain PAO8 as recipient there was no cotransduction of *hutU108* with *met-28* or *ilv-202* (Table 2). The value obtained for cotransduction of *met* and *ilv*, selecting for *ilv*$^+$, was 37% compared with 28% reported by Pemberton & Holloway (1972*a*). These results would suggest a map order *hutU*(*C*)–*ser-3*–*ilv-202*–*met-28*. It has been shown that *hutC* is closely linked to *hutU* (Potts, 1975) but the relative position of the two genes is not known.

Table 2. *Linkage of* hutU108 *to* ser-3

	Recipients	
	PAO2 *ser-3 hut*$^+$	PAO8 *ilv-202 met-28 hut*$^+$
Selected marker	*ser*$^+$	*ilv*$^+$
No. of transductants tested	200	100
No. *hut*$^-$	31	0
No. *met*$^+$	—	37
Linkage		
hutU108–ser-3	16%	—
hutU108–ilv-202	—	0%
met-28–ilv-202	—	37%

The donor lysate of F116 was prepared on PAO1632 *hutU108 ser*$^+$ *ilv*$^+$ *met*$^+$.

Strain PAO1879 is a *hut*$^-$ mutant but the gene defect has not been identified biochemically. Plate mating suggested that the wild-type allele of this *hut* gene entered earlier than the *hutU* gene and an interrupted mating experiment gave it a time of entry of 18 min (Fig. 3).

3. DISCUSSION

We have shown that the genes for the catabolic enzymes required for the utilization of proline, histidine and acetamide are widely scattered on the chromosome of *Pseudomonas aeruginosa*. The map positions as judged by time of entry in interrupted mating experiments are probably reasonably accurate for the *hut* and *put* markers and for *hutUC* the map position fits very well both for time of entry and for transductional linkage with the *ser-3*, *ilv-202*, *met-28* region. In *P. putida* the *hut* genes determining the enzymes of the pathway *hutH* (histidase), *hutU* (urocanase) and *hutF* (formiminoglutamase) and the regulatory gene *hutC* are linked in a single cluster. We have not yet studied the linkage of other structural genes for histidine utilization in *P. aeruginosa* but histidase and urocanase are co-ordinately regulated and *hutC* is linked to *hutU* (Potts, 1975).

The value of 65–75 min for the entry time of the amidase genes may be an under-

estimate and may need to be revised when more genes have been mapped in this region and detailed linkage analysis is possible.

It was interesting to find that these catabolic genes were at very distant sites on the *P. aeruginosa* chromosome since Wheelis & Stanier (1970) and Leidigh & Wheelis (1973*a*, *b*) have shown by transductional linkage that there is clustering of genes concerned with various groups of enzymes required for the catabolism of aromatic compounds together with the genes required for histidine degradation. This suggested that it would be interesting to investigate whether catabolic genes of *P. aeruginosa* showed any clustering and these results show clearly that this is not so for the *ami*, *hut* and *put* genes. We have found previously with PAC strains that no cotransduction occurs between the amidase genes and genes for acetate or propionate catabolism or any of the auxotrophic markers we tested. This is the first report of chromosomal location of catabolic genes in *P. aeruginosa* and we intend to investigate the map position of other catabolic markers.

We are grateful to Professor B. W. Holloway for very generous provision of strains for this investigation and for advice and helpful discussions which took place in London and in Melbourne. M.D. was in receipt of a Research Training Grant from the Medical Research Council and J.R.P. was on leave of absence from Glaxo Research Ltd.

REFERENCES

Betz, J. L., Brown, J. E., Clarke, P. H. & Day, M. (1974). Genetic analysis of amidase mutants of *Pseudomonas aeruginosa*. *Genetical Research* **23**, 335–359.

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**, 145–153.

Brammar, W. J. & Clarke, P. H. (1964). Induction and repression of *Pseudomonas aeruginosa* amidase. *Journal of General Microbiology* **37**, 307–319.

Brammar, W. J., Clarke, P. H. & Skinner, A. J. (1967). Biochemical and genetic studies with the regulator mutants of the *Pseudomonas aeruginosa* amidase system. *Journal of General Microbiology* **47**, 87–102.

Clarke, P. H. & Tata, R. (1973). Isolation of amidase-negative mutants of *Pseudomonas aeruginosa* by a positive selection method using an acetamide analogue. *Journal of General Microbiology* **75**, 231–234.

Day, M. & Clarke, P. H. (1974). Interstrain transduction in *Pseudomonas aeruginosa*. *Proceedings of the Society for General Microbiology* **1**, 63.

Holloway, B. W. (1975). In *Genetics and Biochemistry of Pseudomonas* (ed. P. H. Clarke and M. H. Richmond). John Wiley, London.

Holloway, B. W., Egan, J. B. & Monk, M. (1960). Lysogeny in *Pseudomonas aeruginosa*. *Australian Journal of Experimental Biology* **38**, 321–329.

Holloway, B. W., Krishnapillai, V. & Stanisich, V. (1971). *Pseudomonas* genetics. *Annual Review of Genetics* **5**, 425–446.

Leidigh, B. J. & Wheelis, M. L. (1973*a*). Genetic control of the histidine dissimilarity pathway in *Pseudomonas putida*. *Molecular and General Genetics* **120**, 201–210.

Leidigh, B. J. & Wheelis, M. L. (1973*b*). The clustering on the *Pseudomonas putida* chromosome of genes specifying dissimilatory functions. *Journal of Molecular Evolution* **2**, 235–242.

Loutit, J. S. & Marinus, M. G. (1968). Investigation of the mating system of *Pseudomonas aeruginosa* strain 1. II. Mapping of a number of early markers. *Genetical Research* **12**, 37–44.

Pemberton, J. M. (1974). Size of the chromosome of *Pseudomonas aeruginosa* PAO. *Journal of Bacteriology* **119**, 748–752.

Pemberton, J. M. & Holloway, B. W. (1972*a*). Chromosome mapping in *Pseudomonas aeruginosa*. *Genetical Research* **19**, 251–260.

Pemberton, J. M. & Holloway, B. W. (1972*b*). A mutant of *Pseudomonas aeruginosa* with increased conjugational ability. *Australian Journal of Experimental Biology* **50**, 577–588.

Potts, J. R. (1975). Histidine catabolism in *Pseudomonas aeruginosa*. Ph.D. Thesis, University of London.

Stanisich, V. A. & Holloway, B. W. (1969). Conjugation in *Pseudomonas aeruginosa*. *Genetics* **61**, 327–339.

Wheelis, M. L. & Stanier, R. Y. (1970). The genetic control of dissimilatory pathways in *Pseudomonas putida*. *Genetics* **66**, 245–266.