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

V. The effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on a donor strain

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1. INTRODUCTION

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) has been shown to be an efficient mutagen for bacteria (Adelberg, Mandel & Chen, 1965) and has been used as a mutagen for the sex factor of *Escherichia coli* by Cuzin & Jacob (1967). NG has also been used to produce auxotrophic mutants of *Pseudomonas aeruginosa* (Loutit, Pearce & Marinus, 1968) and its efficiency was high since in some experiments as many as 2% of the survivors of NG treatment were found to be auxotrophic mutants (M. G. Marinus & J. S. Loutit, unpublished data). For these reasons NG was selected for use in the present work in an attempt to produce donor strain (FP^+) mutants of *P. aeruginosa* which could transfer only one of the two linkage groups reported by Loutit & Marinus (1968) or which had an increased ability to transfer genetic markers.

2. MATERIALS AND METHODS

The original strains of *P. aeruginosa*, the methods of isolation of strains and the 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 are listed in Table 1.

(i) *Treatment with mutagen*

Logarithmic phase cultures of OT 15 were produced in nitrate Nutrient Broth (NB) as described by Loutit, Pearce & Marinus (1968). The cells were deposited by centrifugation, resuspended in the same volume of 0·1 M citrate buffer (pH 5·5) and subjected to NG at a final concentration of 0·1 mg/ml for 20 min at 37 °C. The treated cells were washed once, diluted and plated on brain–heart agar to give single colonies after further incubation. The colonies were then transferred to marked positions on fresh plates (52/plate) and these stock cultures were kept at 4 °C after a further 24 h incubation. All strains showing significant differences from OT 15 in subsequent maleness tests were restreaked and stock cultures were established from single colonies on nutrient agar slopes.

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(ii) *Maleness testing of NG-treated isolates*

In certain experiments the maleness testing was carried out on solid media with the males transferred by replica plating. In other experiments the testing was carried out in liquid culture and both methods have been reported by Loutit, Marinus & Pearce (1968). OT 100 (*ilvB112pro-4leu-1*) was used as recipient and transfer of *ilv*$^+$ and *pro*$^+$ was followed. These are the first markers on the two linkage groups reported by Loutit & Marinus (1968). Mating time in liquid medium was restricted to 20 min, which was adequate for these early markers. Streptomycin was used for contra-selection of the donor strains.

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

Strain number	FP	*str*	Auxotrophic markers	Derivation
OT 1	+	r	*trp-1*	Original donor
OT 2	–	s	*leu-1*	Original recipient
OT 8	+	r	*trp-1ade-2*	Mutant of OT 1
OT 15	+	s	.	Male recombinant from OT 8 and OT 56
OT 44	+	s	*met-6*	Mutant of OT 15
OT 47	–	r	*ilvB112leu-1*	Recombinant (Loutit Pearce & Marinus, 1968)
OT 56	–	s	*lys-1leu-1*	Mutant of OT 2
OT 93	–	r	*his-2*	Mutant of OT 47 (3 steps)
OT 100	–	r	*ilvB112pro-4leu-1*	Mutant of OT 47
OT 302*	+	s	.	Mutant of OT 15

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

* This strain was isolated in the present investigation following NG treatment of OT 15.

(iii) *Infection of strains with the sex factor (FP)*

Since all strains to be infected were sensitive to streptomycin this could not be used for contra-selection. Instead, the donors and recipients were distinguished nutritionally and two donors were used for this reason. The prototrophic donor OT 15 was used for those recipients which were auxotrophic and an auxotrophic derivative OT 44 was used for the recipients which were still prototrophic. The donor and recipient cells were prepared and mated as described by Loutit, Pearce & Marinus (1968). The mixture was left for 80 min, diluted 10^{-1} in minimal medium and shaken on the Mickle shaker. It was then further diluted and plated on brain–heart agar to give single colonies on incubation. The colonies were tested on minimal agar to distinguish original donors and recipients, and 120 of the latter were patched on brain–heart agar plates and tested for maleness by replica plating on a lawn of OT 93 (*his-2*).

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(iv) *Quantitative testing of male cultures*

The donor and recipient cells were prepared and mated as described by Loutit, Pearce & Marinus (1968). Mating was carried out for 80 min and the mixture was diluted 10^{-3} in minimal medium before separating the mated cells on the Mickle shaker. Samples (0·2 ml) were plated in triplicate to select for *ilvB112*$^{+}$ recombinants and for some experiments further dilutions and platings were made to isolate colonies from cells of the recipient population on streptomycin agar.

The number of recombinants was scored after 3 days' incubation and related to the number of donor cells in the original mixture. At least 120 of the colonies from the recipient population were tested for maleness by replica plating.

3. RESULTS

(i) *Qualitative differences between treated male cultures*

OT 15 was the donor strain used for these investigations and, in the first experiments, cells were subjected to NG and some 700 treated isolates were examined for maleness using the replica plating method with OT 100 as recipient. In this way qualitative differences in the ability to transfer the early markers (*ilvB112* and *pro-4*) of the two linkage groups could be demonstrated. Only 17 of the isolates were different and, of these, two transferred only one marker and 15 were unable to transfer either.

On subsequent examination it was shown that the two strains which transferred one marker had an auxotrophic block at the other site. When tested for ability to transfer markers other than *ilvB112* and *pro-4* they appeared to be the same as OT 15. The fifteen strains which did not transfer either marker were assumed to be cured of the sex factor and an attempt was made to reinfect them with FP. This procedure divided the isolates into two groups. One group of nine was able to be reinfected and each isolate was presumably cured of the sex factor by the NG treatment. The other group of six could not be reinfected and this result cannot be accounted for at present. One possibility is that they contain sex factors which have mutated and are no longer able to act as sex factors but render the cells immune to further infection.

(ii) *Quantitative differences between treated male cultures*

In further experiments OT 15 was again treated with NG and approximately 500 isolates were tested for maleness in liquid cultures using OT 100 as recipient and testing for the transfer of *ilv*$^{+}$ and *pro*$^{+}$. Cultures of OT 15 were included and any isolate showing greater ability than OT 15 to transfer either or both linkage groups was selected for further study. From the 500 strains three were selected and of these only one (OT 302) showed significantly greater transfer of the two early markers. This strain produced about double the number of recombinants per 10^{3} donor cells compared with OT 15 tested under identical conditions. Two attempts have been made to enhance the donor ability of the new strain OT 302 by treating with NG and examining 500 colonies but they were not successful.

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To determine whether the improvement in OT 302 was due to a mutation in the sex factor or in the organism itself it would be necessary to transfer the sex factors of OT 15 and OT 302 into separate cultures of the same recipient and test the resulting strains for their donor abilities.

(iii) *Comparison of the sex factors in strains OT 1, OT 15 and OT 302*

As a preliminary to this investigation quantitative figures were obtained for the three strains showing their respective abilities to transfer the marker *ilvB112* as well as the sex factor. Knowing that stored cultures lose some of their ability to act as donors, each of the strains was plated out and at least twelve colonies were tested quantitatively for donor ability following culture in nitrate NB (Loutit & Marinus, 1968). The best culture from each strain was selected and subcultured twice in nitrate brain–heart broth to further enhance donor ability (Loutit, Pearce & Marinus, 1968). The three donor cultures were then mated with cells from a single suspension of strain OT 100 as described. The results are shown in Table 2 and it is clear that there is a real difference between the three donors. The original strain OT 1 is much less efficient than the others and OT 302 was consistently better than OT 15. It might be imagined that the differences were due to variations in growth rates, since the least efficient donor was an auxotroph and OT 302 could have had a growth rate greater than OT 15 to account for its increased efficiency. In fact, OT 1 and OT 15 have identical growth rates and OT 302 is significantly slower than either and there is no explanation at present for the differences between the strains.

Table 2. *Comparison of donor abilities* of three male strains of* Pseudomonas aeruginosa *as measured by their ability to produce* ilvB112^{+} *recombinants and to transfer FP*

Donor strain	Recombinants per 1000 donor cells	Males per 100 recipient cells
OT 1	0·22	2·2
OT 15	3·2	11·8
OT 302	8·7	22·0

* Mating was for 80 min and the recipient was OT 100. The results are the mean of two experiments.

Attempts were then made to transfer the sex factors of the three donor strains (OT 1, OT 15 and OT 302) to cells of OT 2. After 80 min mating, colonies from cells of the recipient populations were examined and 17 of 81, 12 of 120 and 2 of 180 were found to be infected by the sex factors of OT 302, OT 15 and OT 1 respectively. The new donor cultures were then tested for maleness in liquid culture and were shown to be poor males similar to OT 1. The method is sufficiently sensitive for preliminary screening and clearly shows differences between the three donors (OT 1, OT 15 and OT 302) but unfortunately there is no way to express the differences. To provide this, quantitative experiments were carried out with at least two of the new strains from each donor. For comparison the original donors

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were tested but without any preliminary selection of more active colonies. All donors were subcultured twice in nitrate brain–heart broth before use. Only the ability to produce *ilvB112*+ recombinants was determined and the results are shown in Table 3.

Improvement in donor ability of OT 302 does not appear to have resulted from a mutation in the sex factor and the results suggest that in the three original donor strains, the efficiency of donor ability was determined by the bacterium in which the sex factor resided rather than the factor itself.

Table 3. *Comparison of donor abilities* of three male strains of* Pseudomonas aeruginosa *and three new male strains prepared from them by transferring their sex factors to cultures of the same recipient strain. Donor ability was measured by the production of* ilvB112+ *recombinants*

Donor strain	Recombinants per 1000 donor cells
OT 1	0·1
OT 2 infected with FP from OT 1	0·3
OT 15	2·0
OT 2 infected with FP from OT 15	0·3
OT 302	3·6
OT 2 infected with FP from OT 302	0·4

* Mating was for 80 min and the recipient was OT 47. The results are the mean of at least two experiments. With the freshly infected strains similar results were obtained with at least one other isolate.

4. DISCUSSION

Treatment of OT 15, a donor strain of *P. aeruginosa*, with the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine has resulted in strains with different characteristics from the original.

One group of strains no longer transfers chromosome markers and has been divided further into those apparently cured of sex factor, since they were able to be reinfected, and another group which could not be reinfected. The curing of the first group was unexpected since previous attempts to cure donor strains of *P. aeruginosa* with acridine orange had been unsuccessful (Holloway & Fargie, 1960; Pearce, 1965; Loutit, unpublished data). There have been, however, reports of curing *E. coli* with NG, and Willets (1967) thought that elimination of F*lac* by NG was due to a mechanism involving mutagenesis. Possibly the same mechanism is involved here since the cells were not grown in the presence of NG and were washed and plated out immediately following treatment.

The strains which could not be reinfected were of interest since they could also have arisen by mutation of the sex factor. The existence of such a mutation could be proved by demonstrating that the cells still had some residual male activities or by showing that the defective sex factor could be transferred into new recipients which would then be unable to act as a recipient. With *P. aeruginosa*, however, proof is difficult to obtain since there are no demonstrable characters associated with the sex factor other than the ability to transfer itself or promote chromosome

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transfer. There are no male-specific bacteriophages and in this laboratory we have not been able to demonstrate any male-specific antigen, even with strains like OT 302 (Pearce, 1965; Loutit, unpublished data). In addition there are no characters like drug resistance or colicinogeny which would make the transfer of the sex factor easy to detect. Nevertheless, an attempt was made to show transfer of the defective sex factors. The defective strains were mated with a suitable recipient for 80 min and 200 recipient cells were isolated and allowed to grow into colonies. Each colony was then tested for its ability to act as recipient and it was found that all of them had retained this ability. From this it was assumed that the defective sex factors could not be transferred but the possibility remains that they could be transferred with an efficiency very much less than that of OT 1. Since each recipient colony had to be tested individually the search was not continued but it would be interesting to do so if simpler detection methods were available.

Only one strain showed enhanced activity and this was investigated further to determine whether it had resulted from a mutation in the sex factor. Since it is not yet possible to get 100 % transfer of sex factor in *P. aeruginosa* and the original strain OT 1 gave only 1 % transfer of sex factor in 80 min, it seems likely that the system of conjugation is subject to repression (Meynell, Meynell & Datta, 1968). With this as a working hypothesis it was assumed that OT 302 had resulted from a mutation causing derepression. The results show, however, that the change was one in the bacterium itself as was the enhancement of donor ability in OT 15. This strain was produced by recombination between a derivative of OT 1 and OT 56 (Loutit, Pearce & Marinus, 1968) and it is likely that the significant change in OT 56 was the change to resistance to the virulent bacteriophage E 79 which was used for contra-selection in earlier experiments (Loutit & Pearce, 1965). This point has not been established unequivocally but it has been observed that other FP-infected phage-resistant recipients are better donors than FP-infected OT 2. Attempts have been made to isolate a true derepressed mutant by the method of Edwards & Meynell (1968) but with no success as yet. This is not surprising as there is no quick way of screening large numbers of cells for the transfer of the sex factor and the process is likely to take some time. Nevertheless the present work does suggest that it is possible to obtain mutant sex factors of *P. aeruginosa* and derepressed strains should be able to be isolated if the system is subject to repression. Such mutants would greatly enhance further studies on the process of conjugation in *P. aeruginosa*.

SUMMARY

N-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of a donor strain of *Pseudomonas aeruginosa* resulted in the isolation of strains which were presumably cured of their sex factors, strains which possibly contained mutant defective sex factors and one strain which showed enhanced donor activity. The latter was apparently due to a change in the bacterium and the sex factors of OT 302 and OT 15 were both shown to be similar to the original in OT 1 when the three were transferred into the same strain.

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