*Genet. Res., Camb.* (1970), **16**, *pp.* 179–184
*Printed in Great Britain*



# Investigation of the mating system of *Pseudodomonas aeruginosa* strain I

## VI. Mercury resistance associated with the sex factor (FP)

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(*Received* 13 *April* 1970)

SUMMARY

Resistance to mercury has been shown to be associated with the presence of the sex factor (FP) in *Pseudomonas aeruginosa*. The resistance is expressed in all infected cells even though they do not necessarily function as donors.

For selection of freshly infected recipients when streptomycin or nalidixic acid were used for contra-selection of the donors, the level of mercury had to be reduced to between M/20000 and M/40000 when 500 units of streptomycin were present per ml and to M/40000 when 1·0 mg of nalidixic acid was included in each ml of medium. Normally donor strains would grow in the presence of Hg ions at a concentration of M/2000, which is 10–20 times greater than the concentration in the two selective media.

The kinetics of transfer of the sex factor were investigated and it was shown that transfer began almost as soon as the cells were mixed and was completed in 20 min when 5 min were allowed for pairing.

## 1. INTRODUCTION

The possibility of isolating mutants of the sex factor of *P. aeruginosa* was discussed by Loutit (1969) and Holloway (1969) and it was pointed out that the task was difficult because there was no easy way of recognizing or selecting strains infected with FP. There were no characteristics associated with the sex factor such as drug resistance or aeruginocinogeny and no male-specific antigens or bacteriophages have been detected. The only way to show the presence of the sex factor was to demonstrate the transfer of chromosomal markers, and the methods were generally tedious and not in any way selective.

Novick (1969) suggested that all plasmids are likely to have some associated characteristic, and perhaps the best known one is fertility, which is definitely plasmid controlled. By far the most common characteristic, however, is resistance to physical, chemical and biological agents, and Novick produced a long list of such agents and the plasmids which conferred resistance. From this list a selection was made of those agents which might act on *P. aeruginosa* and these are shown in Table 1. Others in the list were either not applicable or were known not to be effective with *P. aeruginosa*.

The present paper is concerned with attempts to find some character other than fertility to recognize and possibly select strains of *P. aeruginosa* infected with FP. The agents listed in Table 1 were tested against $FP^+$ and $FP^-$ strains looking for any differences in susceptibility.

Table 1. *Selected physical and chemical agents for which resistance is known to be associated with the presence of a plasmid**

| Agent | Plasmid |
|---|---|
| Ultraviolet irradiation | Col I, Col B, R(i), R(f) |
| Arsenate | P plasmids |
| Bismuth ($Bi^{3+}$) | P plasmids |
| Lead ($Pb^{2+}$) | P plasmids |
| Cadmium ($Cd^{2+}$) | P plasmids |
| Mercury ($Hg^{2+}$) | P plasmids, R factors |
| Cobalt ($CO^{2+}$) | R factors |
| Nickel ($Ni^{2+}$) | R factors |
| Sodium dodecyl sulphate | F, R factors |
| Acridines | P-lac, R(f) |

* From Novick (1969).

## 2. MATERIALS AND METHODS

The strains of *P. aeruginosa* used were listed by Loutit (1969). OT 15 and OT 302 were used as $FP^+$ and OT 47 and OT 56 as $FP^-$ strains. Both of the $FP^+$ strains were streptomycin-sensitive, as was OT 56. OT 47 was streptomycin-resistant. The media used have been described by Loutit, Pearce & Marinus (1968) except that the complete agar (BHA) usually contained 2% agar and 2% Brain Heart Infusion. This is an increased concentration of agar to prevent the spreading of colonies, and it should be noted that the concentration is much less than that usually recommended for Brain Heart Infusion (3·7%). The infection of strains with FP has been described by Loutit (1969), the methods for establishing the kinetics of transfer by Loutit, Pearce & Marinus (1968) and the testing for maleness by Loutit, Marinus & Pearce (1968).

### (i) *Preparation of gradient plates*

Gradient plates were prepared by pouring the first layer of 10 ml of BHA with the plates appropriately tilted and, when set, a second 10 ml of BHA containing 20 mg of the particular agent under test was added and allowed to set with the plate horizontal. Substances which were not very soluble were evenly dispersed in the agar before pouring. After drying, the plates were inoculated across the gradient with a loopful of an overnight nitrate Brain Heart Broth (BHB) culture of the $FP^+$ and the $FP^-$ strains and the plates were incubated overnight at 37 °C.

### (ii) *Sensitivity to ultraviolet light*

This was tested by inoculating a loopful of the four cultures to be tested on a quadrant of a BHA plate. Four plates were prepared and one was irradiated for

15 sec, one for 30 sec, one for 45 sec and one for 60 sec using a lamp at a distance which gave a dose of 16 ergs/mm$^2$/sec. The plates were irradiated in a darkened room and were incubated overnight in the dark.

(iii) *Determination of levels of resistance to chemical agents*

Levels of resistance were determined on solid media. A series of twofold dilutions of the agent to be tested was prepared from a stock solution and suitable volumes were added to molten supplemented minimal or Brain Heart Agar (20 ml). After mixing, the contents were poured into individual plates and allowed to set. Each plate within a set was divided into five sections. The inocula were prepared from overnight cultures of the strains in nitrate BHB. The cultures were diluted $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ and one loopful of the original culture and of each of the dilutions was spread on a sector of each plate within a set. The plates were incubated overnight and the growth was compared with that on a similarly inoculated plate containing no additive.

## 3. RESULTS

(i) *Sensitivity to physical and chemical agents*

There was no difference between the FP$^+$ and the FP$^-$ strains in their sensitivity to ultraviolet light.

The following substances were used in gradient-plate sensitivity tests: sodium arsenate, bismuth nitrate, lead nitrate, cadmium chloride, cobaltous chloride, nickel sulphate, mercuric chloride, sodium dodecyl sulphate, and acridine orange. Of these, only three showed any inhibition of *P. aeruginosa* and only one showed any difference between FP$^+$ and FP$^-$ strains. Cobaltous chloride and nickel sulphate affected both types equally but mercuric chloride showed a marked difference and the FP$^+$ strains were much more resistant than the FP$^-$ ones.

(ii) *Levels of resistance*

Because mercury inhibition is likely to be affected by substances in the medium the levels were determined in two different media (minimal and Brain Heart Agar). The other important factor was the size of the inoculum, since protective materials could be present in effective concentrations in larger inocula. Complete inhibition was recorded when the heaviest inoculum (one loopful of an overnight broth culture spread over approximately 1 sq. in.) was inhibited.

In BHA complete inhibition of the FP$^+$ strain (OT 15) was observed at M/500 $HgCl_2$ and normal growth appeared to occur at M/2000. On the other hand the FP$^-$ strain (OT 47) was completely inhibited by $HgCl_2$ at M/64 000 and showed normal growth at M/256 000. In minimal medium supplemented with isoleucine, valine and leucine to allow the growth of the auxotrophic FP$^-$ strain, the FP$^+$ strain was completely inhibited by $HgCl_2$ at M/8000 and gave normal growth at M/32 000. The FP$^-$ strain was completely inhibited by $HgCl_2$ at M/64 000 and gave normal growth at M/256 000. Thus there is little difference in the sensitivity of the

$FP^-$ strain on the two media but the $FP^+$ strain is more sensitive on the minimal medium. For growth on the surface of BHA plates a concentration of M/10000 $HgCl_2$ was selected as suitable for the selection of $FP^+$ strains.

(iii) *Expression of mercury resistance in an $FP^+$ population*

If the mercury was to be effective in selecting all $FP^+$ cells in a population, mercury resistance must be apparent in all infected cells even though it is known that only 10–20 % of the cells in cultures of OT15 and OT302 are capable of acting as donors and transferring the sex factor. This was tested by diluting a stationary phase culture of OT15 and carrying out a viable count plating in both normal BHA and BHA supplemented with M/10000 $HgCl_2$. There was no significant difference between the two counts so that the mercury resistance is expressed in all cells whether or not they can function as donors.

(iv) *The detection of freshly transferred sex factors*

Having established that all cells in a donor population were resistant to mercury it was still necessary to show that the method could be used for the detection of freshly infected recipients plated immediately after mating, on BHA containing $HgCl_2$. A mating was carried out between strains OT302 and OT47 and after 30 min the cells were diluted, separated by shaking and plated on two different media. One was BHA supplemented with streptomycin (500 units/ml), which would select for all the recipients, and the other was supplemented with streptomycin and $HgCl_2$ at M/10000 to detect the FP-infected recipients. At least 100 colonies which developed on the streptomycin plates were patched on fresh plates and tested for maleness by replica plating on a lawn of OT93 (*his-2*). They were also patched on a mercury plate and it was found that there was 100 % correlation between the two methods. From the percentage of positives it was possible to calculate the number in the original sample and compare it with the number of colonies on the plates used for direct selection. The numbers did not correlate and there was a considerable reduction on the direct selection plates. Subsequently it was found that there was good correlation provided that the direct selection plates containing 500 units/ml of streptomycin were made with $HgCl_2$ at concentrations between M/20000 and M/40000. At these concentrations the medium could be used for direct selection and M/25000 was used for subsequent experiments. There definitely seems to be a synergistic effect and this was also noticed in experiments where nalidixic acid was used for contra-selection. With nalidixic acid at 1 mg/ml, $HgCl_2$ had to be reduced to M/40000.

(v) *The kinetics of transfer of the sex factor*

The time of entry of the sex factor was determined in a mating between OT302 and OT47. Five minutes were allowed for pairing and the cells were diluted $10^{-2}$ in nitrate Nutrient Broth. Samples were removed 7, 10, 15, 20, 30 and 40 min after mixing and, after a further $10^{-3}$ dilution, 0·1 ml volumes were plated on BHA supplemented with streptomycin alone and BHA supplemented with streptomycin

and M/25000 $HgCl_2$. After incubation the colonies on each type of medium were counted and the percentage of $FP^+$ cells was calculated at each time. As a further check the colonies on the streptomycin agar were tested for maleness by replica plating and the percentage of $FP^+$ cells was determined independently. The figures are shown in Table 2 and the two methods give similar results. Transfer apparently began at 5 min and was not complete until approximately 20 min after mixing. This agrees with the figure established by Loutit, Pearce & Marinus (1968).

Table 2. *Kinetics of transfer of the sex factor of* Pseudomonas aeruginosa *in a mating* between OT 302 ($FP^+$) and OT 47 ($FP^-$)*

(The sex factor was demonstrated in the recipients by direct selection of mercury resistant colonies and by replica plating of a number of recipients. The results of the two methods are compared.)

| | | Time from beginning of mating (min) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7 | 10 | 15 | 20 | 30 | 40 |
| No. of recipients* ... | | 152 | 139 | 158 | 145 | 154 | 148 |
| A | No. of mercury-resistant recipients† | 3 | 9 | 17 | 20 | 23 | 24 |
| A | Recipients $FP^+$ by direct selection (%)‡ | 2 | 6 | 11 | 13 | 16 | 17 |
| B | No of recipients examined for maleness by replica plating | 250 | 204 | 156 | 104 | 104 | 104 |
| B | No. of recipients $FP^+$ by replica plating | 6 | 14 | 22 | 16 | 16 | 22 |
| B | Recipients $FP^+$ by indirect selection (%) | 2 | 7 | 14 | 16 | 16 | 22 |

* Mating was permitted for 5 min and the cells then diluted $10^{-2}$ in nitrate Nutrient Broth. At intervals thereafter, appropriately diluted samples were plated on BHA + streptomycin to select recipient (OT47) bacteria, and on BHA + streptomycin + $HgCl_2$ (M/25000) to select recipients which had received the sex factor. The results of this direct selection are shown in A. These results were checked by testing recipient colonies isolated on BHA + streptomycin for the inheritance of FP, by crossing with another $FP^-$ strain by replica plating. These data are shown in B and will be seen to correlate closely with those in A.

† Numbers are the mean of two plates inoculated with an 0·1 ml sample.

‡ Percentages are based on a mean of 150 recipients per plate.

## 4. DISCUSSION

The development of a new method for recognizing the presence of the sex factor of *P. aeruginosa* and a method for selecting donor strains should be of value in future investigations of the system. It should facilitate the isolation of mutant sex factors which are necessary to further out understanding of the relationship between sex factor and chromosome and for the development of new strains necessary for further chromosome mapping.

The reasons for the resistance which plasmids confer on their hosts are not clearly understood, but Novick (1969) suggested that they probably erect an accessibility barrier due to either an enzyme inactivation or decreased permeability of the cell or a subcellular component. With mercury resistance there is

another possibility that there is an increased production of a sulphydryl-containing compound which would combine with the mercury ions and render them inactive. At present it is not possible to make any decision as to the nature of the resistance of *P. aeruginosa* infected with FP.

It is worth noting that the mercury resistance is expressed in all infected cells whether or not they are capable of acting as donors. Loutit (1969), and Holloway (1969) suggested that fertility in *P. aeruginosa* is normally repressed, so that it is apparent that the two characters are not controlled co-ordinately. The regulation of different characters controlled by a single plasmid has been discussed by Novick (1969) and he gave examples of plasmids in which all characters are controlled by a single repressor as well as those in which different characters are controlled independently. As an example of the latter control Novick & Roth (1968) showed that both penicillinase production and arsenate resistance in a strain of *Staphylococcus aureus* infected with a plasmid were controlled independently since they were inducible with separate substrates. On the other hand resistance to cadmium, lead and mercury ions was not inducible and was expressed immediately. A further example was provided by Watanabe (1967) when he reported that HFRT (high-frequency resistance-transferring) strains of *Escherichia coli* K12 lost their donor competence after about five generations although they still retained the R factor and the drug-resistant phenotype. Thus it is not surprising to find independent control of two characters by the plasmid sex factor of *P. aeruginosa*.

This work was carried out in the Department of Molecular Biology at the University of Edinburgh. I am extremely grateful to Professor Hayes for his advice and encouragement and for providing facilities. I would also like to thank Mr E. E. Moody for his helpful advice.

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