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Genetic effects of acridines on *Pseudomonas aeruginosa*

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1. INTRODUCTION

One of the important features of bacterial plasmids is that they may be deleted from the genome of the bacterium without undue effects on viability. The elimination of a plasmid can often be achieved by selective chemotherapy, the most effective of such agents being the acridines. The acridines have been found to have a specific inhibitory effect on the transfer of both episomes and chromosome during conjugation in *Escherichia coli* and it has been possible to draw conclusions from such experiments on the nature of DNA synthesis during conjugation (Cuzin & Jacob, 1966). The acridines thus constitute a highly useful means of characterizing particular aspects of the function of episomes in the bacterial cell.

In *Pseudomonas aeruginosa* a conjugation system has been described (Holloway, 1955, 1956; Stanisich & Holloway, 1968) which displays some features in common with that found in *E. coli*. One difference appears to be the refractory nature of the sex factor FP of *P. aeruginosa* to curing by acridines. A series of acridine compounds including proflavine, acriflavine, acridine orange and euflavine used under a variety of conditions were ineffective in eliminating FP (Holloway & Fargie, 1960) even though the infectious character of FP had been demonstrated (Holloway & Jennings, 1958). This inability to cure sex factor may be related to the natural resistance of *Pseudomonas* to this class of compounds (Albert *et al.* 1945) or to some intrinsic difference in the *Pseudomonas* plasmid from those of other bacteria.

The mechanism by which FP controls fertility and its relationship to chromosome transfer in *P. aeruginosa* is not as yet clearly defined and it was hoped that the study of the effect of acridines on conjugation experiments might provide useful information on this problem. Following the development of an interrupted mating system for $FP^+ \times FP^-$ matings (Stanisich & Holloway, 1968) techniques became available for studying the effect of acridines on both the FP transfer from infectious males and chromosome transfer.

* Present address.

2. METHODS AND MATERIALS

The bacterial strains used were as follows:

Strain numbers	Genotype
PAO 60	*ilv-60*, FP^-
PAO 67	*his-67*, *ese-2*, FP^-
PAO 286	*met-28*, *trp-6*, FP^-
PAO 289	*met-28*, *trp-6*, *ese-2*, FP^-
PAO 292	*met-28*, *trp-6*, *str-2*, FP^-
PAO 381	*leu-38*, *str-2*, FP^+
PAO 664	*pro-4*, *pur-66*, *ese-2*, FP^-
PTO 13	*trp-6*, FP^+
PTO 30	*trp-6*, FP^-
PAT 307	*met-307*, FP^+
PAT 404	*his-404*, FP^+
PAT 420	*pur-420*, FP^+
PAT 421	*pur-420*, *ese-2*, FP^+
PAT 904	*his-404*, FP^{+*}
PAT 905	*his-404*, *ese-2*, FP^+

* PAT 904 is a mutant derived from PAT 404 and has different mating properties (see text); PAT 905 is derived from PAT 904.

Abbreviations: *his*, histidine; *ilv*, isoleucine plus valine; *met*, methionine; *pro*, proline; *pur*, purine; *trp*, tryptophan; *ese-2*, resistance to phage E 79; *str-2*, resistance to streptomycin. PAO numbers—derived from strain 1. PAT numbers—derived from strain 2, PTO numbers—recombinants from PAO × PAT.

Media. Minimal Medium (MM): that of Vogel & Bonner (1956) solidified where necessary with 1·5% Difco Bacto Agar. Nutrient Broth (NB): Difco nutrient broth containing 0·5% Difco Yeast Extract. Nutrient Agar (NA) is NB solidified with 1·2% Davis Agar. Heart Infusion Glucose Broth (HIBG): Difco Heart Infusion Broth plus 0·2% glucose.

Infectious transfer of sex factor

Male and female bacteria were grown overnight in NB at 37 °C. Five ml of the male cells were mixed with 0·25 ml of female cells in 5 ml. NB giving a male to female ratio of between 10 and 20 to 1. The mixture was incubated overnight at 37 °C with gentle aeration and the female cells re-isolated by plating appropriate dilutions on media which select the female strain only. Where possible streptomycin-resistant females and streptomycin-sensitive males were used so that females would be readily re-isolated on media containing streptomycin. Colonies from such plates were picked into 2 ml NB and tested for maleness.

The techniques for interrupted mating and for testing for maleness were the same as those described previously (Stanisich & Holloway, 1968).

Acridine solutions

Solutions of acriflavine (AF) (British Drug Houses Ltd., Lond.), ICR 170 and ICR 191 (Dr H. Creech, Institute of Cancer Research, Philadelphia) were prepared in sterile distilled water and stored at 4 °C in the dark.

Method for curing

Nutrient broths at pH $7{\cdot}4 \pm 0{\cdot}2$ and containing various concentrations of the acridine compound were inoculated with log phase cells to a final concentration of 50–100 cells/ml. These were incubated aerated at 37 °C until the cell density increased to *ca.* 10^6/ml. Serial subculture to fresh acridine broth was continued using a similarly low cell inoculum. Single colonies isolated on NA at each subculture were tested for curing by the spotting method.

3. RESULTS

A number of different types of male strains can be distinguished in *P. aeruginosa*. Strain 2 is a naturally occurring male strain which can transfer chromosome to the female strain 1 at a frequency varying for different markers from 10^{-5} to 10^{-8}. Some of this variation is due to DNA host specificity differences. We have not been able to detect the transfer of the sex factor FP from strain 2 to strain 1 in the absence of chromosome transfer. Some recombinants of strain 1 × strain 2 crosses acquire the sex factor FP, the frequency depending on the marker selected. These 1 × 2 recombinants which have acquired FP can transfer chromosome to strain 1 at about the same or slightly higher frequency to that in 1 × 2 crosses. In addition, they can also transfer FP to strain 1 at frequencies up to 70–80 %, independently of any detectable chromosome transfer, this latter type of sex factor transfer being analogous to the infectious transfer of F in *Escherichia coli*. The strain 1 derivatives which have acquired FP infectiously in this way transfer chromosome to the normal (FP^-) strain 1 at frequencies of 10^{-4}–10^{-5}, but can transfer FP independently of chromosome only at very low frequency (less than 1 %). Thus males in *P. aeruginosa* are defined mainly by their ability to transfer chromosome and the various types differ in their ability to transfer the sex factor FP independently of such chromosome transfer.

(i). *The effect of ICR nitrogen half mustards on maleness*

The ability of ICR Nitrogen half mustards to cure *E. coli* F^+ *rec*$^-$ strains which are normally refractory to curing with the standard acridine orange treatment has been reported by R. C. Clowes & E. E. M. Moody (personal communication) while Ames & Whitfield (1966) have described the high mutagenic ability of these compounds in *Salmonella*. In view of the powerful activity of these compounds it seemed profitable to test their plasmid curing ability in *P. aeruginosa*. The two nitrogen half mustards used were ICR 170 and ICR 191 and the procedure for curing was as described above. Hirota (1960) had observed that an important

prerequisite for curing F^+ strains of *E. coli* was that the strain could transfer the sex factor infectiously. Consequently the strain thought most suitable for the study of acridine curing was the infectious recombinant male PTO 13 which can transfer FP independently of chromosome. After exposure of the strain to these two compounds at concentrations of 25 and 50 μg/ml, single clones were isolated and tested for maleness by the spotting technique. From many hundred colonies examined, one clone obtained after incubation with 50 μg/ml ICR 191 gave no recombinants with the female tester. All the other clones tested did give recombinants, although usually at approximately half the control frequency.

Table 1. *Fertility changes in a* Pseudomonas aeruginosa *male following treatment with nitrogen half mustards*

Cross	Recombinants/2×10^8 male cells
PTO 13 (FP^+) × PAO 67 (FP^-)	1500
PTO 13 (FP^+) × PAO 381 (FP^+)	0
PAO 381 (FP^+) × PAO 67 (FP^-)	2000
PTO 30 × PAO 67 (FP^-)	0
PTO 30 × PAO 381 (FP^+)	950

The infectious male PTO 13 and its ICR 191-treated derivative PTO 30 were mated with male and female derivatives of strain 1. The cell density of saline suspensions of log phase cells was adjusted to *ca.* 2×10^9 cells/ml, 0·1 ml of the mating mixture plated onto MM and the plates incubated at 37 °C for 48 h.

Plate matings with both male and female tester strains were carried out with this particular isolate, designated PTO 30. The results of these matings are given in Table 1. It is seen that the treated strain now recombines only with the male tester and not with the female tester, unlike PTO 13 from which it was derived. These results demonstrate that the treated strain is acting as acceptor of genetic material and consequently that its mating type has been altered from the FP^+ to the FP^- state. It was subsequently shown that a streptomycin-resistant mutant of this derivative could be re-infected with sex factor by mixed culture with PTO 13, thereby regaining its ability to transfer chromosome to an FP^- strain with the original frequency of the parent strain.

In considering the nature of the change in PTO 30, it must be remembered that many experiments in *P. aeruginosa* have been carried out using a variety of acridines and other chemicals under a variety of conditions and in no case has any such clear modification of the mating characteristics of a male strain been reported. In addition, the very low recombination frequency of $FP^+ \times FP^+$ crosses suggests that spontaneous loss of the FP factor is extremely low. We are certain that the isolation of PTO 30 is not due to a spontaneous event. However, the term 'cure' as applied to episomes in other bacteria is probably not correct. Perhaps it is better to conclude that ICR 191 has produced a mutation of FP which has led to its inability to replicate, with the production of a strain which is thus FP^- in phenotype.

Following this successful conversion of PTO 13 to such a female state, experiments involving the ICR compounds were extended to the other male strains of *P. aeruginosa*. Strain PAO 381, which normally exhibits only low infectivity of FP, was chosen as representative of the strain 1 males. It was found that curing of this strain could not be achieved with either compound, although about 10% of the treated strains were found to give a lower recombination frequency than the untreated strain. It was hoped that these strains represented mutated males, and several of them were subjected to further treatment with nitrogen half mustards. However, re-isolated clones gave the same pattern of lower recombination frequency with the female tester and no recombinant formation with male strains, indicating that a complete elimination of the sex factor, as occurred with PTO 30, had not been achieved. In view of the low infectivity of FP in such strains and the somewhat equivocal nature of the fertility changes, this aspect of the work was not pursued further.

Similar experiments were carried out in strain 2 using a histidine auxotroph PAT 404 and treatment with ICR 191 at a concentration of 100 μg/ml. The strain was serially passaged in broth containing ICR 191 four times and 50–100 colonies tested at each passage. The indicator system chosen to detect cured strains was to spot the treated males onto a lawn of another strain 2 auxotroph, PAT 420 (*pur*$^-$, FP$^+$) on MM. It was anticipated that an FP$^+ \times$ FP$^-$ mating would be appreciably more fertile than FP$^+ \times$ FP$^+$ matings, which are known to give only a very low frequency of recombination (Holloway, 1956) and which do not produce any prototrophic recombinants in this spotting technique.

From 100 clones tested after the third serial passage of PAT 404, two clones gave rise to recombinants with the male tester. However, when purification of one of the clones was attempted by single colony isolation, the resultant clones were found to be heterogeneous in that some produced recombinants with the male indicator while others did not. The behaviour of the latter type was typical of the untreated parental strain. This segregation persisted during several subsequent purification steps, but a culture was eventually obtained in which all derivatives tested gave recombinants with the male indicator. This strain, derived from PAT 404, was numbered PAT 904.

A series of plate matings was carried out to determine the nature of this change in mating ability by PAT 904, and both PAT 404 and PAT 904 were mated with a strain 2 male (grown at 37 °C) and a strain 1 female grown at both 37 and 43 °C prior to mating, the higher incubation temperature being used to overcome any effects on recombination due to restriction, growth of strain 1 at 43 °C producing a semi-permanent restriction-deficient phenotype (Rolfe & Holloway, 1966). The results of these crosses are given in Table 2. It is seen that not only does PAT 904 mate with the strain 2 derivative (i.e. PAT 307) at a markedly increased frequency, it also shows an increased frequency of recombination with strain 1 grown either at 37 and 43° C. This unusual mating behaviour of PAT 904 was further investigated in the following ways.

(1) It was shown by crossing PAT 904 with a range of strain 2 auxotrophs, that

the frequency of prototroph formation varied about a hundred fold depending upon the particular auxotrophic marker selected. This is very similar to the situation in 1 $FP^- \times 1$ FP^+ crosses previously reported (Stanisich & Holloway, 1968). The interpretation adopted for the 1×1 crosses was that initiation of chromosomal transfer occurred only at a few sites on the male chromosome and that 1 $FP^- \times 1$ FP^+ crosses showed some similarities to $Hfr \times F^-$ crosses in *E. coli*. It will now be possible to compare the results of 1×1 and 2×2 crosses in *P. aeruginosa* with particular reference to these chromosomal sites of initiation and determine if they are the same for strain 1 and strain 2 males.

Table 2. *Mating characteristics of a mutant (PAT 904) derived by nitrogen half mustard treatment of PAT 404*

Cross	Recombinants/10^8 cells of PAT 404 or PAT 904
PAT 404 × PAT 307	0
PAT 404 × PAO 602 (grown at 37 °C)	1
PAT 404 × PAO 602 (grown at 43 °C)	15
PAT 904 × PAT 307	174
PAT 904 × PAO 602 (grown at 37 °C)	4
PAT 904 × PAO 602 (grown at 43 °C)	54
PAT 404 × PAO 381	0
PAT 904 × PAO 381	0

Matings were carried out between saline suspensions of the various parents listed above. The cell density of the suspension was adjusted to 2×10^9 cells/ml in each case and 0·1 ml of each parental mixture plated on MM and then incubated at 37 °C for 48 h.

(2) PAT 904 was crossed to PAO 286 (*met try*), selection made for met^+ or try^+ and any resulting *try* or *met* recombinants tested for mating type. As with crosses of PAT 404 to PAO 286, some of the recombinants do become male, as tested by their ability to mate with 1 FP^- auxotrophs. However, there does not seem any difference in the mating characteristics of the male recombinants derived from the PAT 904 × PAO 286 cross to those derived from the PAT 404 × PAO 286 cross.

(3) An experiment was set up to determine the direction of transfer in crosses between normal PAT strains and PAT 904. As has been previously shown (Holloway & Fargie, 1960) the direction of transfer of chromosome can be determined, at least in strain 1 × strain 1 crosses, by making each parent resistant in turn to the virulent phage E 79 then, after mating phage resistant and sensitive parents, the mating mixture is treated with the phage. The results of such crosses with PAT 904 are shown in Table 3. Controls of the method where both parents are either phage-resistant or phage-sensitive are included. It is seen that in PAT 904 × PAT 420 crosses, PAT 904 can act both as a recipient of chromosome in crosses with strain 2, and also as a donor, although the frequency of recombination in the latter case is much lower. The lower recombination values in conjugation for *ese-2* derivatives of strain 2 mutants is a general observation and is not limited to the mutants used in Table 3. Two difficulties which arise in the interpretation

of the data from Table 3 are, first, that, as described above, different auxotrophic markers show different recovery values, which may explain the apparently low ability of PAT 904 to act as donor. Secondly, it is not known how the inheritance of the sensitive allele of the marker for response to E 79 will affect the recovery of prototrophs in this experiment. Both these factors could act to reduce the frequency of recombination, and we conclude from the data of Tables 2 and 3 that PAT 904 may act as both genetic donor and recipient in conjugation in crosses with strain 2 and it certainly acts as a donor in crosses with strain 1.

Table 3. *The donor–recipient relationship in the strain 2 × strain 2 type cross involving PAT 904, PAT 404 and derivatives of these strains resistant to virulent phage E 79*

	Recombinants per 10^7 initial parental cells in mating mixture	
PAT strains mated*	No phage	Phage added
404 × 420	0	—
904 × 420	525	11
904/E × 420	245	32
904 × 420/E	221	39
904/E × 420/E	136	166

* 904/E and 420/E are, respectively, the phage E 79-resistant mutants 905 and 421, of PAT strains 904 and 420.

PAT 904 (*his*$^-$), a fertility mutant derivative of PAT 404 was crossed to PAT 420 (*pur*$^-$). The mating mixture consisted of 1 ml of an exponential phase culture of each parent added to 10 ml HIBG, which was then incubated at 37 °C without shaking for 60 min. To 1·0 ml of this mixture was added 1 ml of the virulent bacteriophage E 79, (or 1 ml of broth as a control) the mixture vortexed for 1 min then allowed to stand at 37 °C for 4 min. Aliquots (0·2 ml) were plated in minimal layers on minimal plates and incubated for 2 days at 37 °C.

(4) An attempt was made to determine whether or not the changed mating properties of PAT 904 were due to a mutation in the sex factor, or a mutation of some locus on the chromosome affecting expression of the sex factor. A mixed inoculum of PAT 904 and PAT 420 was grown in broth overnight, in a manner similar to that used to detect the transfer of the sex factor FP from an infectious strain such as PTO 13 to an FP$^-$ strain 1. The mixture was plated on supplemented minimal plates to select for PAT 420, colonies were picked and tested for their ability to cross to PAT 307. None acquired this property and hence there is no evidence that a mutated sex factor can be transferred in an infectious manner from PAT 904 to a normal PAT strain. Nor could the sex factor from PAT 904 be transferred in this way to PTO 30 or to any strain 1 female strains. However, it was possible to demonstrate the transfer of the mating characteristics of PAT 904 in conjugation. PAT 904 was crossed to other strain 2 mutants which were doubly auxotrophic, and thus singly auxotrophic recombinants could be isolated and tested for their ability to cross to PAT 307. It was found that recombinants from some crosses had acquired this ability. Any ability of strain

2×2 crosses to give recombinants in such a test is significant, as the normal recombination frequency of 2×2 crosses is less than 10^{-8}. However, this pattern of inheritance of mating ability in crosses involving PAT 904 is no different from the normal inheritance of FP in 1 $FP^- \times 1$ FP^+ crosses (Stanisich & Holloway, 1968). As the failure to infectiously transfer a sex factor from PAT 904 to either PAT 420, PTO 30 or to any strain 1 females is inconclusive, we cannot at this stage distinguish whether the mating ability of PAT 904 is the result of a mutated sex factor or a chromosomal mutation affecting the expression of the normal sex factor.

ii. *Effect of AF on infectious transfer of FP*

In view of the low ability of strain 1 males such as PAO 381 to transfer FP independently of chromosome, PTO 13 was used for these experiments, as infectious transfer of FP to FP^- strains can readily be obtained with PTO 13. Infectious

Table 4. *Infectious transfer of FP in the presence of acriflavine*

Ratio $FP^+:FP^-$	Concentration of acriflavine (μg/ml)	% FP transfer 8 h	% FP transfer 16 h	Inhibition %
40:1	0	40	85	97
40:1	50	< 1	2	
10:1	0	—	20	65
10:1	15	—	7	

Infectious transfer experiments were set up using PTO 13 (FP^+) and PAO 292 (FP^-). At the times indicated, the female was re-isolated on nutrient agar containing 250 μg/ml streptomycin and 100–200 clones tested for the presence of sex factor by the plate spotting method.

transfer experiments involving the infectious male PTO 13 and a strain 1 female were carried out in the presence of AF. The results obtained are given in Table 4. It is seen that in the presence of 50 μg/ml AF the transfer of sex factor is markedly reduced. When the AF concentration is reduced to 15 μg/ml the effect on transfer, although diminished, is still marked.

Three effects of AF likely to produce these observed results are:

(*a*) a direct effect on the transfer of sex factor, possibly through inhibition of its replication,

(*b*) an indirect effect by inhibiting pair formation between male and female cells,

(*c*) curing of the converted females at the time of infection with sex factor, or shortly afterwards. Information to distinguish between these various possibilities can be obtained by studying the effects of acridine on the transfer of chromosome during conjugation.

iii. *Effect of AF on recombinant formation*

Interrupted matings were carried out between 1 FP^+ and 1 FP^- strains of *P. aeruginosa* to study the effect of AF on conjugation. The mating mixtures contained AF at a concentration of 15 μg/ml. Table 5 shows the results of inter-

rupted matings using three different female strains and the male strain PAO 381. Early and late entry markers were selected and it is seen that matings carried out in the presence of acriflavine show an almost complete inhibition of recombinant formation. Samples taken even up to 160 min after mixing fail to yield more than a few recombinants.

Table 5. *Recombinant formation following interrupted matings in the presence of acriflavine*

FP⁻ recipient	Selected marker	Entry time (min)	Concentration of acriflavine (μg/ml)	Recombinants per 0·2 ml samples		
				20 min	60 min	120 min
PAO 664	*pro*⁺-*4*	2	0	750	—	—
			15	20	—	—
PAO 67	*his*⁺-*67*	7	0	—	2400	—
			15	—	10	—
PAO 289	*trp*⁺-*6*	30	0	—	—	460
			15	—	—	6

Interrupted matings were carried out as described in Methods using the female strains indicated and the donor strain PAO 381. In the test cross-parental strains were mixed in the presence of 15 μg/ml acriflavine which was excluded from the control cross carried out simultaneously. At the times indicated 0·2 ml samples from each of the matings were plated onto the appropriate selective media subsequent to phage kill of the male. Plates were incubated at 37 °C for 48 h and the recombinants counted.

As with the effects of AF on the infectious transfer of FP, the inhibitory effect of AF on recombinant formation may be due to a variety of reasons:

(*a*) inhibition of pair formation between male and female cells,

(*b*) inhibition of the initiation of chromosome replication postulated to occur prior to and concomitant with chromosome transfer,

(*c*) inhibition of chromosome transfer itself,

(*d*) inhibition of the integration of the transferred genetic material into the female recipient, or an elimination of the transferred material without any effect on integration.

A series of experiments were conducted to determine which, if any of these, was the most likely to explain the results obtained.

(iv). *Effect of AF on the female recipient*

The interrupted mating procedure in *P. aeruginosa* involves plating samples of an undiluted mating mixture at various time intervals. No dilution of the mating mixture can be included due to the lower recombination frequency found in this organism as compared to F⁻ × Hfr crosses in *E. coli*. For these acridine experiments this procedure introduces a possible error due to the carry-over of AF from the mating mixture to the selective media onto which the samples are plated. The inhibition of recombinant formation as seen in Table 5, may be a direct result of the effect of AF on the zygote, the AF acting either on the female recipient by

killing the zygote, by the elimination of the transferred material or by an effect on the integration of the transferred material into the female chromosome.

In order to assess the effect of AF on the zygote, an interrupted mating experiment was carried out using the male strain PAO 381 and a female carrying the *his-67* allele which is transferred early in conjugation (7 min). After sixty minutes mating, samples were plated either onto minimal medium containing 50 μg/ml AF or on the same medium without AF. To ensure that the cells from the mating mixture were immediately exposed to high concentrations of the acridine on plating, the minimal layers used in the plating procedure contained AF.

It was found that plating such a mating mixture onto MM + AF gave rise to equivalent numbers of recombinants to the plating on MM. It was thus concluded that the integration process was not affected by AF and hence that AF inhibits recombinant formation through some action on the male parent.

(v) *Effect of AF on pair formation*

An attempt was made to study the effect of AF on pair formation by a procedure which allowed pair formation to occur in the presence of 15 μg/ml AF, after which a dilution step reduced the acridine concentration to what was assumed

Table 6. *The effect of acridine on pair formation in conjugation in* Pseudomonas aeruginosa

Treatment	Recombinants/10^7 male cells in original mating mixture
5 min to allow for pair formation in HIBG followed, by 1/100 dilution into HIBG for 60 min.	255
5 min to allow for pair formation in HIBG + AF, followed by 1/100 dilution into HIBG for 60 min.	67
5 min to allow for pair formation in HIBG, followed by 1/100 dilution into HIBG + AF for 60 min.	71
5 min to allow for pair formation in HIBG + AF, followed by 1/100 dilution into HIBG + AF for 60 min.	8

Log phase cells of PAO 381 and PAO 67 were mixed in a 1:4 ratio in 20 ml broth with or without 15 μg/ml acriflavine. Pair formation was allowed to occur for 5 min after which 1 ml samples were diluted 100-fold into prewarmed broth with or without 15 μg/ml acriflavine. After 60 min incubation at 37 °C the diluted mixtures were filtered through millipore filters and the cells resuspended from the filters in 2 ml of the virulent phage E 79 (*ca.* 10^{10} p.f.u./ml). After 5 min incubation at 37 °C to allow adsorption, 0·2 ml samples were plated, in layers, onto MM. Plates were incubated at 37 °C for 48 h and the recombinants counted.

to be a negligible level, namely 0·15 μg/ml. Interrupted matings carried out in this low level of acridine give normal entry curves and recombination frequencies, indicating that this concentration is not inhibitory to the conjugation process. After allowing 60 min for chromosome transfer the cells were collected by filtration on a membrane filter, the male cells killed with virulent phage E 79, and samples plated on selective media to detect recombinant formation.

The results of such an experiment are given in Table 6. As with the results given in Table 5, a marked inhibition of recombinant formation is observed where mating takes place entirely in the presence of AF. However, where only the initial five minute period takes place in the presence of AF, recombinant formation is significantly higher although not as high as that observed in the control mating without any AF. While this lower recombinant formation could be due to a partial inhibition of AF on pair formation, we think it more likely that there is an inhibition of chromosome initiation or transfer due to intracellular AF present in the cell even after the dilution step. It may take an appreciable time for this AF to leave the cell and hence, while present it could continue to affect the formation of recombinants. Replicate experiments and matings of different parents under the same conditions gave results consistent with this view. Hence we conclude that at a concentration of 15 μg/ml, AF does not affect pairing and the prevention of recombinant formation must be due to inhibition of chromosome initiation or transfer in the male.

(vi) *Effect of AF on chromosome transfer*

An interrupted mating experiment was carried out using PAO 381 and a doubly auxotrophic female carrying the *met-28* and *trp-6* markers. These markers are transferred in interrupted matings at approximately 30 min. The experimental procedure involved three simultaneous interrupted mating experiments with samples taken from each at regular intervals up to 120 min.

One series acted as control to provide the normal entry curve of the *trp*$^+$*-6* allele. The second and third series were the test crosses and AF was added to them at 30 and 70 min respectively to a final concentration of 15 μg/ml. These series were designed to show the effect of acridine on conjugation *after* the initiation of chromosome transfer had begun, and are therefore expected to provide information on the effects of AF on chromosome transfer. Figure 1 shows the results of this experiment. It is seen that:

(*a*) The rate of recombinant formation is depressed, and in the case of the addition of AF at 30 min, this may cause an apparent later entry of *trp*$^+$*-6*.

(*b*) The maximum level of recombinants produced is lower after AF addition.

These effects could be due to an effect of AF on rate of transfer. If this is the case then it should not matter very much what time we add the AF. But in fact Fig. 1 shows that the addition at 30 min results in an overall greater depression of recombinant formation than at 70 min, and if we add AF at the time of mating, the effects are even more drastic, as shown in Table 5 for the three markers *pro-4* (entering about 2 min) *his-67* (entering about 7 min) and *trp-6* (entering about 30 min).

It thus seems as though AF is affecting the initiation of chromosome transfer. In any mating such as that shown in Fig. 1, we would expect considerable asynchrony with respect to the initiation of chromosome transfer in different mating pairs, hence if AF inhibits chromosome initiation, then with the addition of AF at 30 min only those cells which have initiated transfer are not inhibited

by AF, and hence the total number of recombinants would rise to a plateau, the plateau with AF addition at 70 min being higher than the plateau formed after the addition of AF at 30 min.

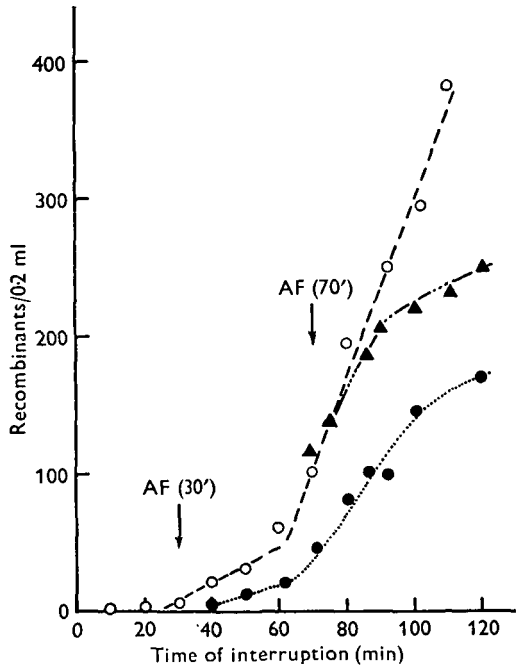

Fig. 1. Effect of AF on interrupted mating. PAO 289 was crossed to PAO 381 and selection made for *trp*+. AF was added to mating mixtures at 30 and 70 min after mating began. Without AF, ○—○; AF added at 30 min ●—●; AF added at 70 min ▲—▲.

4. DISCUSSION

Our previous results have failed to show any effect of acridines such as acriflavine, proflavine, etc. on the sex factor of *P. aeruginosa*. However, the above results on the effect of acriflavin on chromosome transfer and sex factor transfer show that this is not due to any intrinsic comprehensive resistance of *P. aeruginosa* to the action of acridines. The effect of acriflavine on chromosome transfer in *P. aeruginosa* is entirely similar to the effect of acridines on Hfr × F⁻ crosses in *E. coli* and strongly suggests that FP has a similar action in promoting chromosome transfer to that demonstrated for F. The results suggest that under normal vegetative replication the FP factor is acridine-resistant, but that it acquires sensitivity when concerned with the replication of DNA either of itself or of the bacterial chromosome. The objection raised to some of the acridine studies in *E. coli*, namely that the acridine acts not on the transfer of DNA, but through inactivation of the DNA immediately after transfer to the recipient bacteria, does not apply to *P. aeruginosa* in view of its high natural resistance to acridines (Albert *et al.* 1945) and the demonstration by Arai & Watanabe (1967) that acridine-resistant recipient strains of *E. coli* still showed a sensitivity to such

acridines of transfer of episomes and chromosome. In contrast Samaha, White & Herrmann (1967) have described an acridine-resistant sex factor for *E. coli* which is resistant to curing by acridine orange, has unusual transfer kinetics, and shows transfer kinetics which are unaffected by acridine orange.

The action of the ICR compounds in inducing changes, probably mutational, in FP, is encouraging for the future investigation of conjugation in *P. aeruginosa*. Previous efforts by various investigators in this laboratory with both acridines and various mutagens have failed to change the mating behaviour of male strains. Not only have the ICR compounds produced a female mutant of the infectious male PTO 13 but they have produced a fourth class of male strain in PAT 904. This acts as a donor (but not a recipient) for strain 1, and a donor and recipient for strain 2.

The particular interest in this strain 2 mutant is that it may provide an experimental approach for the isolation of an Hfr type mutant in strain 2. Previous attempts to find such a mutant in strain 1 have been unsuccessful, but this may be due to lack of the necessary integration site in the strain 1 chromosome. If Hfr strains in *P. aeruginosa* result from the integration of the sex factor, it is more likely that we will find them in the strain of origin of this sex factor. Electron microscope examination (B. Veitch, unpublished observations) of both PAT 404 and PAT 904 shows no difference in the surface configuration and no structures corresponding to sex pili were seen in either strain.

SUMMARY

Mutants of *Pseudomonas aeruginosa* male strains with altered mating properties have been obtained through treatment with nitrogen half mustards. The response varied with the type of male strain. A mutant of the infectious male PTO 13 was obtained which acts as a female strain and has apparently lost the FP sex factor. A mutant of the non-infectious strain 2 male mates not only with strain 1 females, but shows a thousand-fold increase in its ability to mate with strain 2 males, when it can act as either donor or recipient in conjugation. Derivatives of strain 1 males were obtained which had reduced recombinant forming ability.

While acriflavine is ineffective in producing such mutants, and has not been shown to cure male strains of their sex factor in *P. aeruginosa*, it is very effective in inhibiting infectious transfer of FP from FP^+ to FP^- strains. Furthermore, it markedly inhibits recombinant formation in $1\ FP^- \times 1\ FP^+$ crosses, and this latter effect is thought to be due to the inhibition of chromosome transfer by the male parent. In view of the almost complete lack of effect of AF on the normal growth of *P. aeruginosa* it is likely that the control of chromosome replication during vegetative cell division is different from the occurring during conjugation and that the FP factor is involved in this control during conjugation.

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