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A mutant sex factor of *Pseudomonas aeruginosa*

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SUMMARY

A mutant with the properties of a recipient has been isolated from the *P. aeruginosa* donor strain PAT (FP2+) following treatment with the acridine-mustard ICR-191. While this strain displays the properties expected of a female or recipient in a number of genetic tests, the FP2 determined property of mercury resistance is retained by the strain, suggesting that it may carry the FP2 factor in a mutated form. Treatment of the donor strain PAT (FP2+) with acridine-mustard has produced mutant male strains with the ability to form recombinants with other male strains at frequencies similar to that obtained in FP2+ × FP2− matings. This characteristic has been shown to be due to a mutation in the FP2 factor which is dominant to the wild-type function. The isolation of stable male strains carrying both the mutant and wild type forms of the sex factor suggests that more than one copy of the FP2 factor occurs in *P. aeruginosa* strain PAT donors.

1. INTRODUCTION

Pseudomonas aeruginosa strain PAT (strain 2) behaves as a genetic donor to the unrelated recipient strain PAO (strain 1) (Holloway, 1955, 1956). The agent promoting donor ability in strain PAT is the FP2 sex factor which has been found to have properties in common with sex factors described in the *Enterobacteriaceae*. A proportion of the recombinants (called PTO strains) derived from PAT (FP2+) × PAO (FP2−) crosses inherit donor ability, and the properties of two such male strains (PTO 629 and PTO 13) which can transfer FP2 to FP− strains at high frequency have been described previously (Holloway & Jennings, 1958; Stanisich & Holloway, 1969*b*).

FP2 in strain PAT is refractory to elimination (curing) by agents promoting loss of extrachromosomal elements in other organisms (Holloway & Fargie, 1960; Stanisich, 1968) and this has hindered the development of a conjugation system within this strain. Consequently much of the genetic analysis of *P. aeruginosa* has been confined to strain PAO in which male and female lines are readily available, the former possessing the FP2 sex factor of strain PAT (Loutit *et al.* 1968*a*, *b*, *c*, 1969; Stanisich & Holloway, 1969*a*; Holloway, Krishnapillai & Stanisich, 1971).

Recently, mutant strains acting as recipients in conjugation have been isolated

following treatment of *P. aeruginosa* donor strains with either the acridine half-mustard ICR191 or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Stanisich & Holloway, 1969*b*; Loutit, 1969). Mutants of male strains have also been found which are either defective in sex factor and chromosome transfer (Loutit, 1969), show an increased recombinant formation with female strains (Loutit, 1969) or produce recombinants when mated to either male or female strains (Stanisich & Holloway, 1969*b*). One mutant of this latter type, PAT 904, was characterized by its ability to produce recombinants at similar frequencies in matings with both FP2− and FP2+ lines of strains PTO and PAO, although FP2+ × FP2+ matings normally produce very few recombinants (Holloway, 1956). The lack of a recipient line of strain PAT prevented further investigation as to whether this property of PAT 904 was the result of a mutation in the FP2 sex factor or a chromosomally located gene. In this paper the isolation and properties of such a recipient strain will be described. This strain has been used to demonstrate that PAT 904 possesses both a wild type and mutant form of the FP2 sex factor. A derivative of PAT 904, PAT 906, appears to be homozygous for the mutant FP2 factor.

2. MATERIALS AND METHODS

The bacterial strains used were as follows (Table 1):

PAO strains were formerly designated strain 1, PAT was strain 2.

PTO strains are recombinant lines derived in matings of PAT × PAO.

Antibiotic resistance genes: *chl* = chloramphenicol; *str* = streptomycin.

Amino acid requirement genes: *arg* = arginine; *his* = histidine; *met* = methionine; *pro* = proline; *trp* = tryptophan.

Phage resistance genes: *ese* = resistance to phage E79.

FP2 = strain PAT sex factor.

FP2–1, FP2–2 = sex factor mutants derived from FP2.

Mating phenotypes: FP+ = wild-type donor; FP− = recipient; FP* = donor with 'extended fertility' phenotype.

The temperate bacteriophage F116L (Krishnapillai, 1971, *Molec. Gen. Genet.*, in press) was used for transductions.

Media. Nutrient Broth (NB) was Oxoid Nutrient Broth No 2 (CM67) supplemented with 0·5% Oxoid Yeast Extract (L 21), or Difco Heart Infusion Broth (0038·01) (HIB). Nutrient Agar (NA) was Oxoid Blood Agar Base (CM 55) supplemented with 0·5% Oxoid Yeast Extract.

NA supplemented with 10 μgm/ml $HgCl_2$ was used to test mercury-resistance of FP2+ bacteria.

Minimal medium (MM) (Vogel & Bonner, 1956) was solidified with 1·2% Oxoid Agar No. 1 (L 11) and supplemented when necessary with L-amino acids to a final concentration of 1 mM. X 1 minimal medium, used in the replica plating technique for mating type, was MM supplemented with 2% NB. The antibiotics streptomycin (sulphate) and chloramphenicol were added to MM and NA at various concentrations.

TNM buffer (pH 7·4) contained tris 0·01 M, NaCl 0·15 M, $MgSO_4$ 0·01 M. Preparation of acridine solutions and method for curing were as given in Stanisich & Holloway (1969*b*).

Mating on the plate. Saline suspensions of donor and recipient bacteria, prepared from logarithmic phase cultures in NB, were adjusted to a cell density of *c.* 2×10^9/ml. One tenth ml aliquots of each parent were then spread over the surface of selective MM. If a high recombination frequency was expected 0·1 ml aliquots of a diluted suspension of the male was used; the recipient strain was not diluted. Plates were incubated at 37 °C for 48 h before the recombinants were counted.

Replica-plating for determination of mating type. Colonies to be tested for mating type were patched to NA or supplemented MM and grown at 37 °C for 6 h or until a patch of growth was clearly visible. These patches were then replicated to selective X 1 minimal agar previously spread with a saline suspension (*c.* $1–2 \times 10^9$ bacteria) of either an indicator donor or recipient strain. The plates were incubated at 37 °C for 48 h to allow recombinant formation. If colonies were to be tested against *both* male and female indicator bacteria then duplicate master plates were prepared. This involved replication of the original master plates after 6 h incubation to identical medium, followed by a second incubation period for these two master series.

Infectious transfer of the sex factor FP2. Logarithmic phase cultures (in NB) of FP2+ donor and recipient bacteria were mixed in NB to a cell density of *c.* 5×10^8 cells/ml. The ratio of input cells was usually 10:1 in favour of the donor strain, though other ratios were used. The mixture was incubated without shaking at 37 °C for 4–6 h and then diluted and plated to selective media to reisolate the recipient strain. Clones derived from bacteria receiving the sex factor could be identified by the replicating technique described above.

EMS mutagenesis. Two drops of ethyl methane sulphonate (EMS) were added to 10 ml of bacteria in logarithmic phase (*c.* 5×10^8 cells/ml), vigorously agitated and maintained at 37 °C without shaking for 1 h. One ml aliquots of the treated culture were diluted 1 in 30 in NB and incubated O/N at 37 °C with aeration. Auxotrophs were identified by the replica-plating technique.

3. RESULTS

(i) *Isolation of a recipient line in strain PAT*

The method adopted for the isolation of a recipient line from the male strain PAT, involved the use of the acridine half-mustard ICR 191 at 100 μg/ml using the procedure described by Stanisich & Holloway (1969*b*) for the isolation of a recipient line from the recombinant male PTO 13. The strain chosen for treatment was PAT 404 (*his-404*, *str-1100*, FP2+) and single clones surviving mutagen treatment were tested for recombinant formation with the indicator male strain PAT 458 (*trp-1100*, *str-1100*, FP2+), using the patching technique described in Materials and Methods. The recombination frequency for FP2+ × FP2+ matings

Table 1. *Bacterial strains*

Strain	Genotype	Mating phenotype	Parent	Reference
PAT 2	Prototroph, FP2+	FP+	.	Holloway (1955)
PAT 404	*his-404*, *str-1100*, FP2+	FP+	PAT 3	Stanisich & Holloway (1969*b*)
PAT 458	*trp-1100*, *str-1100*, FP2+	FP+	PAT 3	Fargie & Holloway (1965)
PAT 900	*his-404*, *str-1100*, FP2−	FP−	PAT 404	.
PAT 904	*his-404*, *str-1100*, FP2+, FP2−1+	FP*	PAT 404	Stanisich & Holloway (1969*b*)
PAT 906	*his-404*, *str-1100*, FP2−2+	FP*	PAT 904	.
PAT 919	*his-404*, *arg-1104*, *str-1100*, FP2−	FP−	PAT 404	.
PAT 963	*arg-1104*, *str-1100*, FP2−	FP−	PAT 2 × PAT 919 recombinant	.
PAT 964	Prototroph, FP2−	FP−	PAT 963	.
PAT 967	*met-1105*, FP2−	FP−	PAT 964	.
PAT 973	*met-1105*, FP2+	FP+	PAT 967	.
PAT 975	*met-1105*, *chl-1100*, FP2−	FP−	PAT 967	.
PAT 976	*met-1105*, *chl-1100*, FP2+	FP+	PAT 975	.
PAT 977	*met-1105*, *chl-1100*, FP2−2+	FP*	PAT 975	.
PAT 1218	*pro-1100*, *ese-1106*, FP2+	FP+	PAT 694	.
PTO 13	*trp-6*, FP2+	FP+	PAO 286 × PAT 420 recombinant	Stanisich & Holloway (1969*a*)
PTO 30	*trp-6*, FP2−	FP−	PTO 13	Stanisich & Holloway (1969*b*)
PAO 41	*trp-54*, *str-6*, *chl-13*, FP2+	FP+	PAO 68	Holloway Collection
PAO 68	*trp-54*, *str-6*, *chl-13*, FP2−	FP−	PAO 13	Holloway Collection

of *P. aeruginosa* is of the order of 10^{-7} to 10^{-8}/parent cell (Holloway, 1956) compared to 10^{-2} to 10^{-6} for FP 2+ × FP 2− matings (Holloway *et al.* 1971), thus it was expected that conversion of treated males to the recipient state would show a concomitant increase in recombinant frequency enabling any such changes to be readily detected by the patching technique.

This procedure led to the isolation of two mutant recipient strains out of 700 clones screened from the third serial passage of the PAT male in HIB containing ICR 191 at 100 μg/ml. The properties of one of these isolates, designated PAT 900, will be described below.

(ii) *Analysis of strain PAT 900*

(*a*) *Plate matings*

The parent strain PAT 404 and its derivative, PAT 900, were tested for recombination ability by mating to both a PAT male strain and to the recipient derivative of a PTO line, PTO 30 (Stanisich & Holloway, 1969*b*). The latter strain was grown at 43 °C prior to mating in order to eliminate any restriction effects and hence enhance recovery of recombinants (Rolfe & Holloway, 1966). The results obtained are given in Table 2. It is seen that PAT 900 shows a marked increase in recombinant formation with the strain PAT male, while its recombination ability with the female PTO strain is reduced below that of the parental strain, PAT 404. These findings are consistent with the view that PAT 900 is acting as a genetic recipient and has lost the donor ability characteristic of the parent male strain.

Table 2. *Mating characteristics of strains PAT 404 and PAT 900*

Cross	Recombinants/2 × 10^8 cells of PAT 404 or PAT 900
PAT 404(FP 2+) × PAT 458(FP 2+)	0
PAT 900 × PAT 458(FP 2+)	*c.* 2000
PAT 404(FP2+) × PTO 30(FP 2−) (grown at 43 °C)	100
PAT 900 × PTO 30(FP 2−) (grown at 43 °C)	0

The male strain PAT 404 and its derivative PAT 900, isolated following ICR treatment, were mated with a strain PAT male, PAT 458, and a female line of strain PTO, PTO 30. The cell density of saline suspensions of log phase cells was adjusted to *c.* 1–2 × 10^9 cells/ml and 0·1 ml of each of the parental suspensions plated on MM, then incubated at 37 °C for 48 h.

(*b*) *Direction of chromosome transfer in matings with PAT 900*

Like the parental PAT 404, PAT 900 is resistant to streptomycin but sensitive to the virulent phage E 79. To determine the direction of chromosome transfer, PAT 900 and PAT 404 were mated to a streptomycin-sensitive, E 79-resistant derivative of a PAT male strain, either streptomycin or E 79 being used to contraselect one or the other of the two parental strains involved in each cross. The results of such an experiment are given in Table 3. It is seen that when PAT 900 is killed by E 79 subsequent to mating, there is an almost complete abolition of recombinant recovery suggesting that this strain is acting as a genetic recipient,

while selective kill of the strain PAT male parent has no effect on recombinant production. The control cross of the two males shows the expected low level fertility.

Table 3. *Selective kill of parental strains in matings involving PAT 404 and PAT 900*

Cross	Recombinants/c. 10^8 cells PAT 694 selective procedure		
	None	+Phage	+Streptomycin
PAT 404(FP2+, SM-r, E79-s) × PAT 694 (FP2+, SM-s, E79-r)	4	1	2
PAT 900(SM-r, E79-s) × PAT 694(FP2+, SM-s, E79-r)	c. 4000	50	c. 3000

Two ml of a log phase culture (10^9 cells/ml) of each parent was mixed in 4 ml prewarmed HIB and incubated without shaking at 37 °C for 90 min. The mixtures were then centrifuged and the bacteria resuspended in 2 ml TNM buffer. Aliquots (0·2 ml) were plated on MM (no selection) or on MM containing 1 mg/ml streptomycin (streptomycin selection). In addition, to a 1 ml volume of the TNM suspension was added 1 ml of E79 (*c.* 10^{10} p.f.u.) and the mixture incubated at 37 °C for 10 min to allow adsorption. Aliquots (0·2 ml) were then plated on MM (phage selection). All plates were incubated at 37 °C for 48 h.

(*c*) *Reinfection of PAT 900 with the FP2 sex factor*

Recipient strains isolated following elimination of a sex factor, either as a spontaneous event or following treatment with 'curing' agents, should be capable of being reinfected with the wild-type sex factor to regain donor strain properties. Transfer of FP2 to PAT 900 was achieved by mixing exponential phase cultures of PAT 900 with a strain PAT male auxotroph to a final cell density of *c.* 5×10^8 cells/ml and a 1:10 ratio in favour of the donor strain. Following 4 h incubation without shaking at 37 °C, clones of PAT 900 were reisolated on histidine supplemented minimal medium and subsequently tested for inheritance of sex factor by recombinant formation with the male PAT 458, using the replica-plating technique.

Acquisition of sex factor by PAT 900 would be observed as a loss by those cells of the ability to form recombinants when mated to the indicator male, since FP2+ × FP2+ matings show very low fertility. Of 150 clones tested, 20 failed to produce recombinants with the PAT indicator male, but when tested against the female PTO 30, these produced recombinants at levels similar to that observed with the parental PAT 404. It was concluded that these 20 clones were derived from cells of PAT 900 which had been reinfected with wild-type sex factor.

The three lines of evidence given above conclusively show that PAT 900 is a recipient line derived from the donor parent strain PAT. The properties of this strain may be the result either of a loss ('curing') of the FP2 sex factor, or of mutation of this factor which allows the strain to behave as a genetic recipient while preventing its donor functions. Loutit (1971) has reported that increased resistance to mercuric compounds is associated with the presence of FP2 in male lines of strain PAO. The FP2+ strains PTO 13 and PAT 404 were therefore tested for response to mercuric chloride and as expected were found to be resistant.

PTO 30, the recipient derivative of PTO 13, was found to be sensitive and thus behaved as if it had lost the FP2 factor of its male parent. In contrast, PAT 900 was found to retain the mercury-resistance phenotype characteristic of its male parent strain PAT 404, a result which suggests that even although it acts as a recipient in all known respects, strain PAT 900 may still carry a defective FP2 factor.

However, it cannot be assumed that female (cured) derivatives of strain PAT will necessarily be sensitive to mercury, since such cells may well possess a second determinant which confers mercury resistance. Such a determinant could be located on the chromosome or on another plasmid and mask loss of the mercury-resistance function coded by FP2. In view of this uncertainty, and since it acts as a female in every other respect, strain PAT 900 will be designated FP2– for the purposes of this paper although this may be considered a phenotypic rather than a genotypic designation.

(*d*) *Isolation of a prototrophic line from PAT 900*

To establish a prototrophic line from PAT 900 from which other genetically marked derivatives could be obtained, it was necessary to restore histidine independence and streptomycin sensitivity. The latter was desirable to maintain the use of streptomycin as a contraselective agent in future experiments. The streptomycin locus in strain PAT is cotransducible with a gene involved in tryptophan biosynthesis (Fargie & Holloway, 1965; Waltho & Holloway, 1966), while a gene involved in arginine biosynthesis is cotransducible with the streptomycin locus in strain PAO (Waltho & Holloway, 1966; Isaac, Ong & Holloway, in preparation). PAT 900 was subjected to mutagenesis with ethyl methane sulphonate (EMS) and the mutants so derived screened for auxotrophy to either arginine or tryptophan. One such mutant designated PAT 919 has the genotype *his-404*, *str-1100*, *arg-1104*, FP2– and it has been shown that *arg-1104* and *str-1100* are cotransduced at a frequency of 10% by phage F116L.

Strain PAT 919 was then mated to the prototrophic streptomycin-sensitive male PAT 2, selection being made for histidine-independent streptomycin-resistant recombinants. Strain PAT 963, a *str-1100*, *arg-1104* FP2– derivative from this cross, was transduced at low multiplicity of infection using F116L propagated on PAT 2. Screening of the prototrophs for co-inheritance of streptomycin sensitivity resulted in the isolation of a recipient line designated PAT 964. This strain is non-lysogenic for F116L and has been used as the parent strain in the construction of a variety of genetically marked derivatives.

(iii) *Mutant male lines of strain PAT*

In an earlier study, a mutant male derivative of strain PAT 404 (*his-404*, *str-1100*, FP2+) was obtained by treatment with ICR191 (Stanisich & Holloway, 1969*b*). This mutant, designated PAT 904, was found to differ from its parent in that it gave recombinants in crosses with other males of strain PAT. However, since PAT 904 also gave recombinants in crosses with female lines of strain PAO,

it seemed it had retained the FP2 sex factor (Stanisich & Holloway, 1969*b*). Strain PAT 906 was obtained from PAT 904 following a second treatment of the latter with ICR 191, and was chosen because it showed appreciably more frequent formation of recombinants with the male strain PAT 458.

Table 4. *Comparison of the mating characteristics of PAT 404 and its ICR-treated derivatives PAT 904 and PAT 906*

Test strain	Recombinants/5×10^8 cells		
	PAT 404 (FP2+)	PAT 904	PAT 906
PAT 967 (FP2−)	1140	*c.* 4000	*c.* 10000
PAT 973 (FP2+)	10	620	*c.* 5000
PTO 30 (FP2−) (grown at 43 °C)	22	60	350
PTO 13 (FP2+) (grown at 43 °C)	0	30	800
PAO 68 (FP2−) (grown at 43 °C)	65	150	1500
PAO 41 (FP2+) (grown at 43 °C)	0	30	370

The male strain PAT 404 and its ICR treated derivatives PAT 904 and PAT 906 were mated with male and female lines of strains PAT, PTO and PAO. The cell density of saline suspensions of log phase cells was adjusted to *c.* $1–2 \times 10^9$ cells/ml and 0·1 ml of each of the parental suspensions plated on MM and then incubated at 37 °C for 48 h.

(*a*) *Comparison of the mating properties of PAT 404, PAT 904 and PAT 906*

A comparison of these strains was made using the plate mating procedure in crosses with male and female lines of strains PAT, PTO and PAO. The results (Table 4) show that PAT 404, PAT 904 and PAT 906 have in common the ability to produce recombinants when mated with recipient lines of strains PAT, PTO and PAO. This suggests that the ICR-treated derivatives still retain the donor ability characteristic of the parent line and hence possess the FP2 sex factor determining this property. The three strains differ somewhat in recombination frequency, since strain PAT 906 gives more recombinants that PAT 904, which in turn gives more than the parental strain PAT 404. The ICR-treated derivatives also differ from strain PAT 404 in giving recombinants with male lines of strains PAT, PTO and PAO. Here the increase in recombination frequency over that observed with the parental strain is of the order of a 1000-fold, while once again PAT 906 shows a significantly higher value than that observed with PAT 904. The property of strains PAT 904 and PAT 906 to form recombinants with other male strains will be termed 'extended fertility'.

(*b*) *Transmission of 'extended fertility' to a PAT female strain*

The above mating experiments suggest that PAT 904 and PAT 906 still possess the FP2 sex factor. Experiments were attempted to determine whether the property of extended fertility was the result of ICR mutagenesis of the FP2 factor or of a chromosomally located gene. Table 5 gives the results of an experiment to examine the transfer of sex factor from strains PAT 404, PAT 904 and PAT 906 to an auxotrophic female PAT 967 (*met-1105*, FP2−). The procedure

was as given in Materials and Methods, and derivatives of the recipient population were re-isolated on methionine supplemented minimal medium.

After 24 h incubation at 37 °C two sizes of colonies were found on the selective medium. The larger of the two colony types resembled colonies of the recipient strain PAT 967, which on minimal medium at 18 h incubation form circular, low convex, smooth colonies showing very little variation in colony diameter. On continued incubation to 24 h the colonies tend to spread with the edge becoming crenated and an outer rim of roughness appearing. The smaller of the two colony types were similar in general appearance after 18 h but were 'pin-head' colonies of *c.* 0·3 mm diameter and were rather translucent. On continued incubation these colonies remained compact with an entire edge, but became much more opaque.

Table 5. *Transfer of sex factor to a PAT recipient strain*

Mixture, FP2+ ; FP2−	Form of reisolated clones of PAT 967 on MM	Nos. of clones tested	Recombinant formation with PAT 900 (FP2−)	Recombinant formation with PAT 404 (FP2+)	Mating type
PAT 404+PAT 967	Large	91	+	−	FP+
		489	−	−	FP−
PAT 904+PAT 967	Large	81	+	−	FP+
		5	+	+	FP*
		475	−	−	FP−
	Small	2	+	−	FP+
		150	+	+	FP*
		7	−	−	FP−
PAT 906+PAT 967	Large	6	+	+	FP*
		365	−	−	FP−
	Small	3	−	−	FP−
		189	+	+	FP*

Transfer of FP2 from strains PAT 404, PAT 904 and PAT 906 to the recipient strain PAT 967 was achieved by mixing log phase cultures of male and female bacteria in a 10:1 ratio to a final cell density of *c.* 5×10^8 cells/ml. After 6 h incubation at 37 °C the mixtures were diluted and plated to minimal medium supplemented with methionine to reisolate clones of the PAT 967 recipient. These clones were then tested for recombinant formation with indicator male and female strains PAT 404 and PAT 900 to determine the mating type of the reisolated clones. FP* is used to designate the extended fertility phenotype.

When the large and small clones were tested individually for the inheritance of 'extended fertility' by recombinant formation with male and female tester strains, it was found that this property was almost exclusively (95 %) confined to the small colony type. The frequency of transfer of this property from PAT 904 or PAT 906 could therefore be estimated simply by determining the proportion of small colonies found after 24–36 h incubation on minimal medium. For example, in the experiment shown in Table 5 the percentage of small colonies isolated from the PAT 904 and PAT 906 mixtures was 20 % and 55 % respectively. These results demonstrate that 'extended fertility' is a transmissible property of the ICR treated males and hence the result of a mutation of the FP2 sex factor.

As previously mentioned, mercury resistance cannot be used as a criterion of the presence of the FP 2 factor in strain PAT since both donor and 'recipient' bacteria display this phenotype. However, the property of 'extended fertility' can be transferred from both PAT 904 and PAT 906 to the mercury sensitive recipient line PTO 30. Here the acquisition of 'extended fertility' is found to be directly correlated with acquisition of mercury resistance, supporting the view that extended fertility is the result of a mutation of the FP 2 sex factor. The symbol FP* will be used as a phenotypic designation for the mutant sex factor determining 'extended fertility'.

In a control experiment, transfer of the wild-type sex factor from the parental strain PAT 404 to the recipient PAT 967 occurred at a frequency of 18 % and was not associated with any obvious morphological differences among the colonies formed after 24 h incubation on selective minimal medium.

(*c*) *Heterozygosity of strain PAT 904*

During the initial isolation of strain PAT 904 some difficulty was experienced in obtaining a cell line in which all individual clones produced recombinants when mated to male strains (Stanisich & Holloway, 1969*b*). As well as clones displaying extended fertility, others were found (at appreciable frequencies) which behaved as typical 'wild type' males having the mating characteristics of the parent PAT 404. However, after a series of single clone purification steps a cell line showing less than 0·1 % segregation was obtained. This segregation pattern suggested that 'extended fertility' was possibly associated with a mutation of the sex factor and that the 'wild type' clones represented segregants not receiving the mutant sex factor. This view was not, however, supported experimentally, in that male recombinants isolated from crosses between PAT 904 and a strain PAO female were found to behave as typical FP 2 + males rather than as extended fertility males as would have been expected (Stanisich & Holloway, 1969*b*). The apparent incompatibility between these results and those of the previous section (which indicate that 'extended fertility' is the result of mutation of the FP 2 factor) can be resolved by a further consideration of the data presented in Table 5.

This infectious transfer experiment demonstrates not only that strain PAT 904 possesses a mutant sex factor which confers upon the cells the ability to form recombinants with male strains, but also that wild-type males can be isolated among the recipient population at similar frequencies to those observed with the parent strain PAT 404; that is, at levels of 14 % and 18 % respectively. Since PAT 904 does not show significant levels of spontaneous segregation of wild-type males it is conceivable that this strain is heterozygous for the FP 2 factor, having at least one copy of each of the wild type and the mutant forms, and that these are carried in a stable heritable configuration such that daughter cells at each cell division receive at least one copy of each of the two sex factor forms. Alternatively, PAT 904 may harbour a single recombinant plasmid molecule heterozygous only for that region determining extended fertility and its wild-type homologous sequence. Loss, on transfer, of one or other of these repeated sequences would

satisfactorily explain the two classes of male strain obtained in transfer experiments. This explanation is, however, less likely in view of the results obtained using mutants of FP 2 (FPd mutants) which are defective in sex-factor transfer (Stanisich & Holloway, in preparation). Non-segregating derivatives of these males which are apparently of the genotype FP*FPd, i.e. equivalent to the FP*FP+ heterozygous state proposed for PAT 904, exhibit restoration of FPd transfer function as evidenced by the independent transfer of the FPd factor from the cell. The results previously obtained with the PAO matings (Stanisich & Holloway, 1969*b*) are probably due to a biased selection of recombinants inheriting wild-type FP 2 rather than FP*, particularly since inheritance of the latter is known to be associated with small colony morphology.

The results of sex-factor transfer experiments from PAT 906 (see Table 5) suggest that only the mutant form of FP 2 is present in this strain. Support for this idea was obtained in transfer experiments carried out as for Table 5 except that an interstrain system with PTO 30 as recipient (grown at 43 °C to overcome restriction) was used. The results were qualitatively similar to those obtained with the PAT system, namely transfer of 'wild type' sex factor from PAT 404 and PAT 904 at similar levels (*c.* 2%); no transfer of 'wild type' sex factor from PAT 906, and transfer of the mutant sex factor from PAT 906 and PAT 904. Again transfer of FP* was correlated with small colony morphology, the frequency of such clones being about 20% for PAT 906 and 0·5% for PAT 904.

It is possible that the sex factor mutation determining the 'extended fertility' phenotype is identical in strains PAT 904 and PAT 906. The observed differences in properties may result from strain PAT 904 possessing a 'wild type' FP 2 factor as well as a mutant (FP*) factor, while PAT 906 possesses only the mutant factor. If so, the additional ICR treatment of PAT 904 may have provided the conditions necessary to allow segregation of FP* into a cell line, PAT 906, which is homozygous for this mutant form.

(*d*) *Dominance of 'extended fertility'*

If PAT 904 is heterozygous for two sex factor forms then it follows that the mutation causing 'extended fertility' is dominant to the allele carried by the wild-type sex factor since the 'extended fertility' phenotype is the one expressed in this cell line. As male × male conjugation is possible using PAT 904 and PAT 906 then this property should be transmissible to male strains under conditions allowing infectious transfer of the sex factor.

Table 6 gives the results of an experiment to test for the transfer of sex factor from strains PAT 404, PAT 904, and PAT 906 to a normal male strain PAT 458 (*trp-1100*, *str-1100*, FP 2+). Derivatives of the 'recipient' strain PAT 458 were selected on tryptophan-supplemented minimal medium, and the colonies so obtained were patched and tested for recombinant formation with an indicator male strain using the replica plating technique.

It is seen that 'extended fertility' is transferable from both PAT 904, and PAT 906, although higher levels of transfer are achieved from the latter strain over

short periods of time. As already described for sex-factor transfer to the recipient PAT line, inheritance of the mutant sex factor is associated with the small colony size of the reisolated male 'recipients'. However, when these small clones were streaked to minimal medium they were found to segregate large and small types. By recombinant formation with male and female tester strains it was found that the majority of 'large' clones behaved as normal males (75%) while the 'small' clones behaved as males possessing 'extended fertility'. This segregation of

Table 6. *Transfer of 'extended fertility' to a PAT male strain*

Mixture, FP2+:FP2+, 10:1	% reisolated clones forming recombinants with an indicator male		
	1 h	4 h	8 h
PAT 404+PAT 458	< 1	< 1	< 1
PAT 904+PAT 458	0·25	0·7	50
PAT 906+PAT 458	1	17	50

Log phase cultures of strain PAT 404 (*his-*) and its derivatives PAT 904 and PAT 906 were mixed in HIB at a 10:1 ratio with the male PAT 458 (*trp−*) to a final cell density of *c.* 10^8 cells/ml. At the times indicated samples were diluted and plated to tryptophan supplemented MM to reisolate the 'recipient' 458 strain. Single clones so derived were tested for the ability to form recombinants with another indicator male PAT 404, using the replica plating technique.

male cells receiving the mutant sex factor into two distinct lines suggests not only that 'extended fertility' is dominant to the wild-type allele (since this segregation phenomenon persists through several single clone purifications on minimal medium), but also that an incompatibility exists between the resident wild-type sex factor and the superinfecting mutant form. Similar findings were obtained when *lac*-F+ or Hfr males of *E. coli* were used as recipients in superinfection experiments with *F'-lac* (Scaife & Gross, 1962), or when *F'-lac* males were used to superinfect *F'-gal* recipients (Echols, 1963).

Current views on the mechanism of superinfection immunity (incompatibility) displayed by related sex factors favour the proposal of Jacob, Brenner & Cuzin (1963) that replication of autonomous units requires their attachment to some cell site, perhaps the membrane, and that only a limited number of such sites exist per cell, only one being proposed for the F factor of *E. coli*. Thus the presence of two like-units in a single cell involves a competition between the units for the replication sites available, and if only one such site exists this competition will be manifested by segregation of the units into distinct cell lines since only one is capable of replication at the maintenance site while the other will be diluted out at successive cell divisions. By anology, the segregation of *P. aeruginosa* wild-type males newly infected by FP* donors, into FP* and FP+ cell lines, could be taken as evidence that an incompatibility exists between the wild-type and mutant forms of the FP2 factor, and that this situation is indicative of a low number of attachment sites at which replication of the sex factor can occur. However, the persistent heterozygosity observed in PAT 904 argues in favour of more than one

copy of FP 2 and consequently of more than one maintenance site per cell. This view is further supported by the ease with which stable strains analogous to PAT 904 and possessing two distinguishable forms of the FP 2 factor can be constructed (Stanisich & Holloway, in preparation).

The incompatibility phenomenon observed above is not, however, necessarily inconsistent with the view of multiple copies of FP 2, since competition for a site or sites of replication would presumably still exist between any superinfecting unit and those already established in the cell. This would presumably be manifested as an instability or segregation of either the superinfecting or resident sex factor into distinct cell lines, depending on which unit was successful in competition for the maintenance site. Segration would be expected to continue until the wild-type number of units was re-established in the cell so that a stable, heritable configuration of the multiple copies was restored.

Table 7. *Direction of chromosome transfer in male × male matings*

(Three ml of a log phase culture (5×10^8–10^9 cells/ml) of each parent was mixed in 2 ml of prewarmed HIB and incubated without shaking at 37 °C for 90 min. The mixtures were then centrifuged and the bacteria resuspended in 1·5 ml of TNM buffer. Aliquots (0·2 ml) were plated to MM, and to MM containing 1 mg/ml streptomycin or 750 μgm/ml chloramphenicol and the plates then incubated at 37 °C for 48 h.)

Mating *met* CM-R SM-S + *his* CM-S SM-R	Recombinants/c. 4×10^8 input cells: selective procedure None	+ Streptomycin	+ Chloramphenicol
PAT 975 (FP −) × PAT 900	0	0	0
PAT 975 (FP −) × PAT 404	1500	0	430
PAT 975 (FP −) × PAT 904	3000	0	700
PAT 975 (FP −) × PAT 906	6000	0	692
PAT 976 (FP +) × PAT 900	4000	2000	0
PAT 976 (FP +) × PAT 404	0	0	0
PAT 976 (FP +) × PAT 904	8000	200	175
PAT 976 (FP +) × PAT 906	10000	300	191
PAT 977 (FP*) × PAT 900	8000	6000	0
PAT 977 (FP*) × PAT 404	12000	3000	6
PAT 977 (FP*) × PAT 904	12000	1500	135
PAT 977 (FP*) × PAT 906	16000	1500	162

Recombinant numbers greater than 10^8 are estimates only.

(*e*) *Direction of chromosome transfer in male × male matings*

Strains were constructed from a recipient line of strain PAT to carry either the wild-type sex factor from PAT 404 or the mutant sex factor from PAT 906. These strains were used to determine the direction of chromosome transfer in various combinations of FP + × FP* matings, and in particular to determine the fertility of matings between two FP* donors. Table 7 shows the results of such an experiment where either streptomycin or chloramphenicol were used as selective agents.

It is seen that although male-by-male matings of strains possessing the wild-

type sex factor are of low fertility, matings involving two males possessing the mutant sex factor are of high fertility, producing recombinants irrespective of the selection imposed. This suggests that chromosome transfer can occur to either parent and hence that in populations of these males, cells capable of behaving either as genetic donors or recipients are present. Crosses involving wild-type males with either PAT 904 or PAT 906 are less fertile although again chromosome transfer appears to occur to either parent of the cross. However, since the map positions of the selected and contraselected markers used in these matings are unknown, together with what adverse effects the contraselective procedures may have on recombinant viability, the results obtained are qualitative only, and provide an estimation of whether a particular strain can act either as donor and/or as recipient but does not allow a determination of which event is the preferred or more frequent one.

4. DISCUSSION

The persistence of the FP 2-determined phenotype of mercury resistance in the recipient derivative PAT 900 of strain PAT 404, in contrast to the mercury-sensitive phenotype observed in the PTO 30 derivative of PTO 13, suggests that the former strain may retain the FP 2 sex factor in a defective state. Such a situation could well have been produced by the ICR compound used in these studies as such acridine-mustards have highly mutagenic properties (Ames & Whitfield, 1966) as well as being related to a class of compounds which enhance 'curing' of extrachromosomal elements in bacteria (Hirota, 1960). The plasmid loss induced by a variety of chemical mutagens is thought to be the result of inactivation of genes associated with plasmid replication, thus leading to the segregation of plasmid-less bacteria (Willetts, 1967; Novick, 1969). However, NG can produce variants from *E. coli* F+ cells which behave as recipients, but which still retain some properties of the donor male from which they were isolated, suggesting that the recipient phenotype observed may have resulted from inactivation of certain sex factor functions and that the cells still retain the defective plasmid (Takahashi & Barnard, 1967). The situation observed with strain PAT 900 may be analogous to this. If so, mutation to the recipient state presumably involved loss of at least two donor functions – those of exclusion and of chromosome mobilization.

The ability of PAT 900 to accept and maintain wild-type FP 2, thereby regaining donor functions, does not conflict with the view that it may harbour a defective sex factor, since genetic evidence obtained in experiments with PAT 904 suggest that multiple copies of FP 2 exist in donor bacteria of this strain. Thus incompatibility would not be detected between a superinfecting wild-type sex factor and a defective plasmid which may already be present in PAT 900. For the purposes of genetic analysis, this strain can be considered phenotypically FP 2 –. Evidence distinguishing a 'cured' from a possible defective state must await biophysical studies demonstrating the loss of a satellite band of DNA known to represent the FP 2 sex factor in donor bacteria.

The possibility that two or more copies of the FP 2 factor exist in cells of

P. aeruginosa strain PAT is not in itself an unusual phenomenon and instances can be cited of similar situations among the *Enterobacteriaceae*. Thus Col E1 of *E. coli* is present in at least four copies per chromosome (Bazaral & Helinski, 1968), while plasmid 15 of *E. coli* 15 has 12–15 copies per chromosome (Cozzarelli, Kelly & Kornberg, 1968). However, the F factor of *E. coli* K12 together with some R and col factors appear to be present in the cell in low numbers, 1–2 per chromosome (Freifelder & Freifelder, 1968; Rownd, Nakaya & Nakamura, 1966; Hickson, Roth & Helinski, 1967). The possibility that *Pseudomonas* strains carry multiple copies of FP2 may explain why the FP2 factor appears to be more difficult to 'cure' than the F factor of *E. coli* (Hirota, 1960) or the P plasmids of *Staphylococcus* (Richmond, 1970). Loutit (1969), however, notes a relatively high incidence of suspected FP2− derivatives in *P. aeruginosa* strain PAO following treatment with NG, which may suggest that this strain differs from strain PAT in having only one unit per chromosome.

The mutagenic properties of ICR191 are clearly shown in the isolation of the sex factor mutant conferring the phenotype of 'extended fertility'. This phenotype appears to be dominant to the wild-type state since it is expressed in the heterozygous strain PAT 904 and can be transferred from this strain and from strain PAT 906 in infectious transfer experiments using wild-type males as recipients. Since FP2+ × FP2+ matings in *P. aeruginosa* are of low fertility (10^{-7} to 10^{-9} recombinants/parental cell) it appears that the presence of the homologous wild-type sex factor in the two parental cells prevents either pair formation or subsequent transfer or integration of genetic material. This phenomenon, found generally among plasmid-carrying bacteria, has been termed 'exclusion' (Novick, 1969) and in *E. coli* has been shown to be overcome following growth of F donor bacteria under a variety of cultural conditions (Lederberg, Cavalli & Lederberg, 1952). The F− phenocopies produced lose donor ability and behave as temporary genetic recipients while still retaining the F sex factor. It is seen that the 'extended fertility' mutation in *Pseudomonas* allows FP* cells to overcome the exclusion function (*eex*+) of wild-type males although they themselves behave as phenotypically *eex*− bacteria by accepting chromosomal genetic material from other FP+ or FP* donors (Table 7). The precise alteration in function(s) allowing the expression of the extended fertility phenotype is unknown but may be the result of some change in the structures (pili) suspected to be associated with genetic transfer in *Pseudomonas*, such that effective contact or transfer can now be initiated to other male as well as female bacteria. It is known that the sex pilus *per se* is not responsible for the exclusion phenomenon since mutant male strains producing either no pili or structurally defective pili nevertheless maintain exclusion function (Ohtsubo, 1970; Ohtsubo, Nishimura & Hirota, 1970; Achtman, Willetts & Clark, 1971). However, the implication of exclusion function in some aspect of pilus formation is evidenced by the *tra* J complementation group of transfer-defective mutants of Willetts (1971), which are defective both in pilus formation and exclusion function as the result of a single mutational event (Finnegan & Willetts, 1971). Unfortunately although the recipient ability of *eex*− males is well estab-

lished their ability to act as genetic donors to other wild type or *eex*− males cannot be studied as strains defective in this function alone have not yet been reported. Certainly if F− phenocopies mimic an *eex*− state then such males should act only as genetic recipients in matings with other wild-type males, and not as donors, unless bidirectional genetic transfer can occur once a bridge has been provided by one of the partner donors. This latter possibility is perhaps unlikely and the ability of FP* males to accept genetic material from other donors might best be explained on the hypothesis that they themselves lack exclusion function either as a result of the same or an independent mutation from that which confers upon them the ability to overcome the *eex*− function of wild-type donors.

The increase in recombination frequency observed in matings with both PAT 904 and PAT 906 compared to that obtained with the parent PAT 404 strain (Table 4) might be suggestive of some degree of derepression of the former strains, particularly in view of the small colonial morphology observed among recipient strains newly infected with the FP* sex factor (Table 5). Dowman & Meynell (1970) note a similar phenomenon in strains carrying derepressed col factors, where derepression is associated with a permanent small colonial morphology of these isolates. Males carrying the FP* factor may be less efficient in production of repressor for pilus synthesis than are FP+ males or may have an altered operator site to which repressor binds less readily. This latter hypothesis would seem more probable in view of the increased recombination frequency apparent in strain PAT 904, in which both the wild-type and mutant sex factor forms coexist. Thus transfer of FP* to a recipient line would result in a longer period of derepression than would be expected of FP+ transfer to the same environment.

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