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A new sex factor of *Pseudomonas aeruginosa*

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SUMMARY

Of 150 wild-type strains of *Pseudomonas aeruginosa* examined, 48 formed recombinants when mated to *P. aeruginosa* strain PAO FP$^-$ and hence presumably possess sex factors. Three different types of sex factor were distinguished by the pattern of transfer of particular markers in different regions of the chromosome and by the ability to confer resistance to mercury in strain PAO. One new sex factor, FP39, was studied in detail, and while similar to the previously studied FP2 in terms of transfer kinetics, natural stability and resistance to curing by acridines, it differed from FP2 in promoting chromosome transfer from a site 10 min to the left of the FP2 origin and in showing apparently aberrant entry kinetics for a leucine marker situated 48 min from the FP2 origin. This was due to FP39 having a genetic determinant either for a structural gene of leucine biosynthesis or a specific suppressor gene for this locus. PAO strains carrying both FP2 and FP39 were unstable for both sex factors, suggesting a relationship between them.

1. INTRODUCTION

Conjugation and chromosome transfer in *Pseudomonas aeruginosa* has been shown to be mediated by several plasmids. The best known is FP2 (formerly referred to as FP) (Holloway & Jennings, 1958; Loutit, Marinus & Pearce, 1968; Loutit, 1969*b*; Stanisich & Holloway, 1969*a*; Holloway, Krishnapillai & Stanisich, 1971), but more recently certain R factors have been shown to promote chromosome transfer in this bacterium (Stanisich & Holloway, 1971). The study of plasmids in *P. aeruginosa* has three important aspects – the role of plasmids in conjugation and transfer of genetic material, the contribution of plasmids to the phenotype of host bacteria, and mapping of the *P. aeruginosa* chromosome. Mapping by conjugation using FP2$^+$ × FP$^-$ matings has been of considerable value, but the demonstration that this sex factor promoted orientated chromosome transfer apparently from only a single origin has meant that not all the chromosome was equally accessible to mapping by crosses involving FP2 (Pemberton & Holloway, 1972; Loutit, 1969*a*; Loutit & Marinus, 1969). Accordingly, we have made a survey of wild-type strains of *P. aeruginosa* for other sex factors, primarily

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with the aim of finding any which have a different site of origin. We have found sex factors to occur rather frequently amongst wild-type strains of *P. aeruginosa* and we have characterized some aspects of one new sex factor, FP39.

2. MATERIALS AND METHODS

Bacterial strains. The strains used in this study and their origins are shown in Table 1. Wild-type strains of *P. aeruginosa* used as sources of new sex factors were generously supplied by Dr R. Mushin, Department of Microbiology, University of Melbourne.

Bacteriophage strains. F116, a general transducing phage (Holloway, Egan & Monk, 1960)

Table 1. *Strains used in this study*

Strain no.	Genetic markers	Reference
PAO12	*pur-136*, *leu-8*, *chl-3*, *FP*$^-$	Pemberton & Holloway (1972)
PAO68	*trp-54*, *chl-13*, *str-13*, *FP*$^-$	Stanisich & Holloway (1972)
PAO361	*leu-38*, *str-7*, FP$^-$	This paper
PAO381	*leu-38*, *str-7*, FP2$^+$	Stanisich & Holloway (1969*a*)
PAO664	*pro-64*, *pur-66*, *ese-5*, FP$^-$	Stanisich & Holloway (1969*a*)
PAO886	*pro-71*, *pur-66*, *pyr-21*, *his-151*, *leu-41*, *ese-14*, FP$^-$	Pemberton & Holloway (1972)
PAO1227	*trp-54*, *chl-13*, *str-13*, FP2$^+$	This paper
PAO1231	prototroph, *leu-38*, FP39$^+$	This paper
PAO1235	*trp-54*, *chl-13*, *str-13*, FP2$^+$, FP39$^+$	This paper
PAO1245	prototroph, FP39$^+$	This paper
PAO1260	prototroph, *leu-38*/leu$^+$, FP39$^+$	This paper
PAO1264	*trp-54*, *chl-13*, *str-13*, FP39$^+$	This paper
PAO1376	*pur-154*, *leu-38*, FP$^-$	This paper
PAR39	*pur-136*, FP39$^+$	This paper
PTO13	*trp-6*, *chl-4*, FP2$^+$	Stanisich & Holloway (1969*a*)

The prefix PAO is used to indicate that the strain is derived from strain 1, PAR indicates the strain is a recombinant between PAO1 and another strain, PTO indicates the strain is a recombinant between PAO and PAT (derived from strain 2).

E79, a virulent phage used in interrupted matings (Holloway *et al.* 1960).

Methods. The cultural and other methods used were those described in other publications from this laboratory (Stanisich & Holloway, 1969*a*, *b*; Pemberton & Holloway, 1972). The principal media used included Nutrient Yeast Broth (NYB), Difco Heart Infusion Broth (HIB), Nutrient Agar (NA) (Stanisich & Holloway, 1969*a*, *b*) and Minimal Medium (MM) (Vogel & Bonner, 1956) solidified where necessary with 1% Oxoid Agar No. 1. Interrupted matings were carried out in minimal medium as described elsewhere (Pemberton & Holloway, 1972). Sex-factor transfer procedures were the same as described previously (Stanisich & Holloway, 1972).

3. RESULTS

(i) *Isolation of new sex factors*

The procedure used to isolate new sex factors was to take newly isolated wild-type strains of *P. aeruginosa* and prepare auxotrophic mutants of each strain. Then by using plate matings, each new strain was crossed to selected recipient PAO strains to determine if they could act as donor strains for particular chromosomally located auxotrophic markers. The recipient strains used were PAO12 (*pur-136*, *leu-8*, FP$^-$) and PAO664 (*pro-64*, *pur-66*, FP$^-$) and selection was made in turn for each of the four markers which are in various regions of the known chromosome map of strain PAO. As it is known that DNA specificity differences between conjugating parents can markedly reduce the recovery of recombinants, a restriction-deficient phenotype was induced in the recipient by growth at 43° prior to mating to avoid recombinant loss by this cause (Rolfe & Holloway, 1966, 1969). Of 150 wild-type strains tested by this procedure, 48 acted as donor strains and hence presumably carried a sex factor.

The various donor strains were classified into two groups with respect to their ability to transfer the prototrophic alleles of the various markers of the recipient tester strains PAO12 and PAO664. One group of 28 strains showed a high level of transfer of *pro-64*$^+$ and a low, but equal, level of transfer of both *pur-136*$^+$ and *leu-8*$^+$, thus showing a strong similarity to donors carrying FP2. By contrast, the other group containing 20 strains showed an apparent high level of transfer of *leu-41*$^+$ and a lower level of transfer of *pro-64*$^+$ than that shown by members of the first group.

In order to compare more precisely the properties of those sex factors carried by the 48 donor strains to each other and to the well-characterized FP2, the sex factor from each donor strain was transferred to PAO68, an FP$^-$ strain. The sex factors retained the same donor properties in this new genetic background with respect to the transfer of *pro-64*$^+$, *leu-8*$^+$ and *ade-136*$^+$.

Furthermore, it was found that they were heterogeneous with respect to their ability to confer mercury resistance to strain PAO. Loutit (1971) has shown that the sex factor FP2 carries a genetic determinant which confers increased resistance to mercuric salts in strain PAO. Only four of the 48 donor strains tested grew on NA containing 40 μg/ml $HgCl_2$, and all were similar to FP2 with respect to their ability to promote transfer of *pro-64*$^+$, *leu-8*$^+$ and *ade-136*$^+$.

Some of these donor strains therefore carry sex factors distinguishable from FP2. It was decided to characterize one in detail, and after preliminary testing, FP39 was selected as being different from FP2 with respect to its donor ability for the markers tested in the preliminary screening, and in not carrying a determinant for mercury resistance. FP2 and FP39 were shown to be similar, with respect to stability (when carried by PAO68), and in their resistance to curing by acridines.

(ii) *Comparison of sex factors FP2 and FP39*

(*a*) *Interrupted matings using the FP39 sex factor*

Interrupted matings were carried out using PAO886 as recipient and PAO1227 (FP2$^+$) and PAO1264 (FP39$^+$) as donors; the entry curves obtained are shown in Figs 1 and 2. It is seen that with the exception of *leu-41*$^+$, all the markers transferred from the FP39 donor entered at about 10–12 min later than when FP2 was the sex factor promoting transfer. In addition, not only was the apparent time of entry of *leu-41* very much earlier with FP39 than with FP2, but the frequency of recovery of recombinants with the Leu$^+$ phenotype was considerably higher.

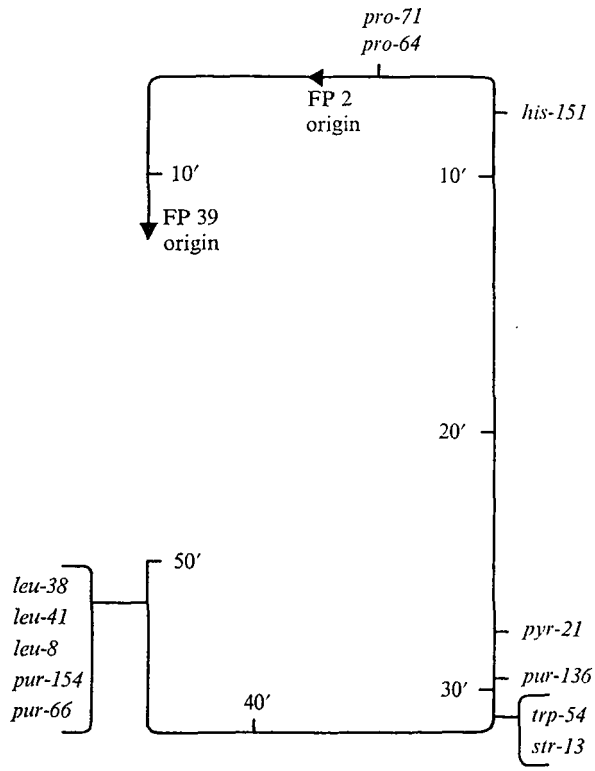

Fig. 1. Abbreviated chromosome map of *P. aeruginosa* strain PAO showing relative position of markers used in this study.

Considering the known chromosome map of *P. aeruginosa* (Fig. 3) (Holloway, Krishnapillai & Stanisich, 1971; Pemberton & Holloway, 1972), it is seen that one possible explanation of this data is that the chromosome is circular, and that FP39 is promoting entry of the chromosome from a site close to *leu-41* and in an anticlockwise direction. Furthermore, the distance between *leu-41* and the FP2 origin would then be about 10 min.

(*b*) *The inheritance pattern of* leu-41 *with FP2 and FP39 donors*

Further data has shown that such an interpretation is incorrect and a more likely explanation is that the sex factor FP39 carries a genetic determinant for leucine synthesis or a suppressor gene for *leu-41*, thus giving a Leu$^+$ phenotype to strains carrying *leu-41*; the apparent inheritance of *leu-41*$^+$ in matings involving FP39 donors therefore results from the inheritance of the sex factor FP39 itself.

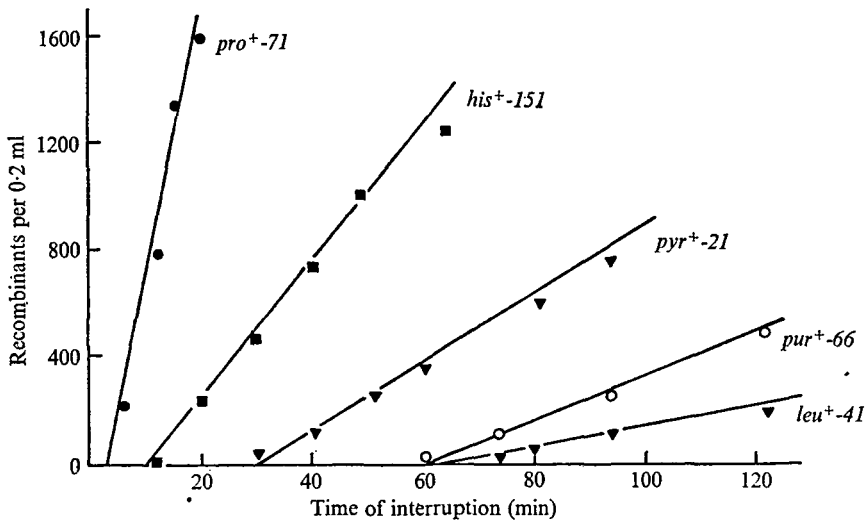

Fig. 2. Times of entry of markers carried by PAO886 in interrupted mating using PAO1227 (FP2$^+$) as donor.

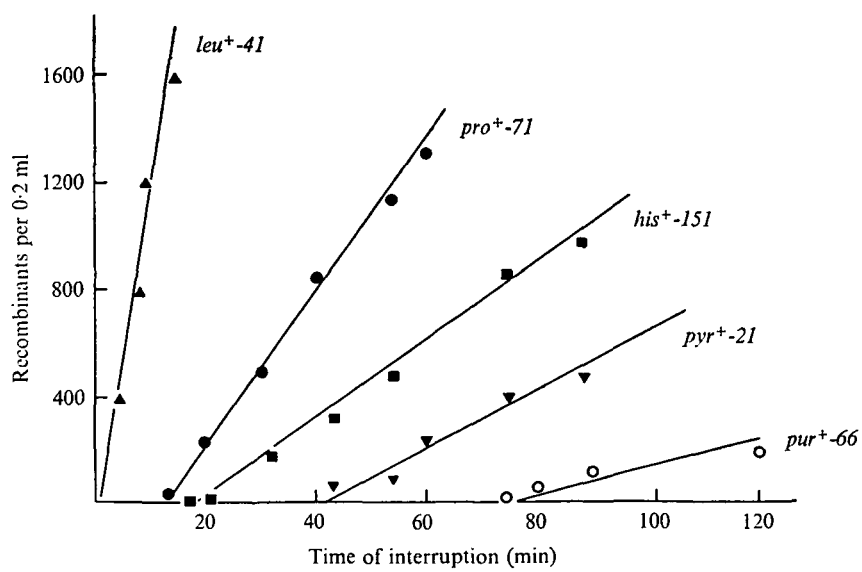

Fig. 3. Times of entry of markers carried by PAO886 in interrupted mating using PAO1264 (FP39$^+$) as donor.

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Fig. 4 shows that the sex factors FP2 and FP39 are transferred from donor strains to a PAO recipient at frequencies which are probably not significantly different. However, with FP39 there is concomitant inheritance of a Leu$^+$ phenotype and the sex factor FP39 when *leu-38* is a marker of the FP$^-$ recipient (*leu-38* and *leu-41* are closely linked, independently isolated mutations of the same *leu* gene situated at about the 48 min site on the chromosome). No such parallel inheritance of FP2 and a Leu$^+$ phenotype occurs with the FP2 donor.

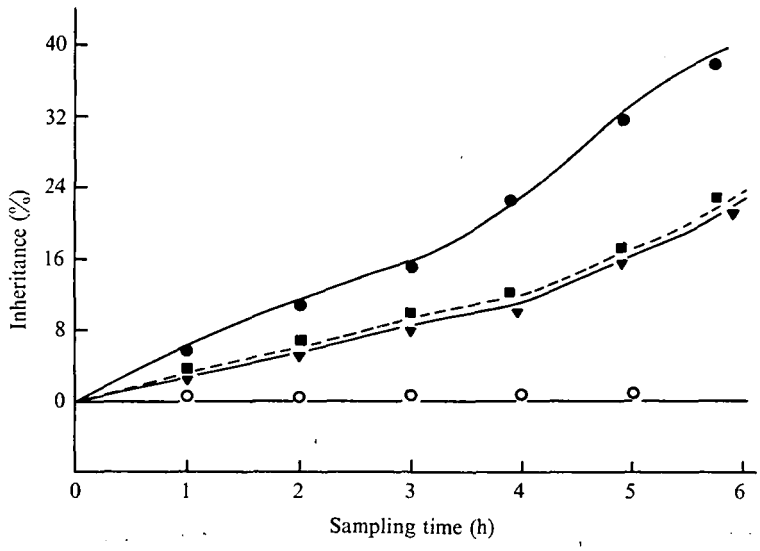

Fig. 4. Kinetics of transfer of FP2 (from PTO13 FP2$^+$) and FP39 (from PAR39 FP39$^+$) to an FP$^-$ *leu-38* recipient (PAO361). The coinheritance of the Leu$^+$ phenotype with each sex factor is also shown. ●, FP2 transfer. ▼, FP39 transfer. ■, Inheritance of Leu$^+$ with FP39. ○, Inheritance of Leu$^+$ with FP2.

This pattern of coinheritance of FP39 and the Leu$^+$ phenotype suggested that either the inheritance of the sex factor was closely associated with the inheritance of that region of the chromosome carrying the *leu-38*, or that a determinant for Leu$^+$ was carried on FP39. Two lines of evidence suggest the latter.

FP39 was transferred from PAO1264 to PAO361 and selection made for Leu$^+$ colonies. One such strain, PAO1231, was then used as the donor in transduction with phage F116, using PAO664 as the recipient. This latter strain carried the marker *pur-66*, which is cotransducible with *leu-38* (and hence with *leu-41*) at a frequency of 20%. If the FP39 Leu$^+$ recombinants are in fact merozygous for *leu-38*, carrying a Leu$^+$ determinant in the sex factor and a *leu-38* allele in the chromosome, the presence of the latter allele can be determined by its linkage to *pur-66*$^+$. Phage F116 propagated on PAO1231 was used to transduce PAO664, selecting *pur-66*$^+$ recombinants. It was found that 20% of the colonies obtained were leucine-requiring, indicating that PAO1231 still carries the *leu-38* allele. Hence PAO1231 was not Leu$^+$ through integration of *leu-38*$^+$ into the chromo-

some, but presumably through some genetic determinant carried by the FP39 sex factor.

PAO1231 was again used as a donor in transduction using F116, but in this case PAO1376 (*leu-38*, *pur-154*, the latter being an independently isolated mutation closely linked to *pur-66*) was the recipient. Here there was no recovery of the *leu-38*$^+$ marker amongst the *pur-154*$^+$ transductants. This is additional evidence to support the view that the Leu$^+$ phenotype of PAO1231 does not result from integration of the *leu-38*$^+$ allele into the chromosome at a site linked to *pur-66*.

Hence we conclude that the FP39 sex factor carries a genetic determinant which enables strains carrying *leu-38* to acquire a Leu$^+$ phenotype when they acquire the sex factor and that strains such as PAO1231 still carry the *leu-38* allele at the normal site in the chromosome.

A reasonable conclusion from this data is that FP39 promotes chromosome transfer from a site 10–12 min proximal to the site of chromosome transfer by FP2. However, as no markers have yet been identified between the site of FP39 and the site of FP2, an equally possible explanation is that FP39 promotes chromosome transfer from the same site as FP2 but that there is a delay of 10–12 min before chromosome movement commences. This possibility thus raises the question as to whether FP2 and FP39 show any relationship, as might be expected if they show the same site of origin.

(c) *Are FP2 and FP39 related?*

The properties of surface exclusion and incompatibility can be used to demonstrate relationships between plasmids (Novick, 1969). Surface exclusion can be shown by a reduced ability of a strain carrying one plasmid to acquire another related plasmid. It was found that PAO381 (FP2$^+$) and PAO361 (FP$^-$), isogenic strains except that the former possessed the sex factor FP2, showed considerable difference in their ability to acquire the sex factor FP39 from the donor PAR39 (FP39$^+$, Str^s). A ratio of 100:1 of 'donor':'recipient' cells was used, mating pairs were allowed 5 min to form, then 0·1 ml of the mating mixture was plated on minimal plates supplemented with 1000 μg/ml streptomycin to select for Leu$^+$Str^r progeny. In the mating PAR39(FP39$^+$) × PAO381(FP2$^+$) there was no recovery of Leu$^+$ clones, while for the mating PAR39(FP39$^+$) × PAO361 (FP$^-$), Leu$^+$ clones were recovered at a level of $1/10^5$ donor cells. The most likely explanation of this result is that FP2 in the recipient has excluded FP39 and hence provides evidence of a relationship. There is no evidence (Stanisich & Holloway, unpublished observations) that FP2 or FP39 affect host-controlled modification in PAO and hence restriction can be excluded as an explanation of the results. An additional possibility is that FP39 was not excluded but prevented by incompatibility from e stablishing itself within the strain-carrying FP2.

By contrast, similar experiments showed that FP2 could be introduced into recipients containing FP39 at much the same frequency as FP$^-$ recipients. No explanation can be given for this non-reciprocal behaviour of FP2 and FP39. However, such behaviour enabled a test of incompatibility of FP2 and FP39 to

be made. Where two sex factors do not exist and function independently of each other in the same cell they are said to be incompatible and this may be evidence of similarity.

A strain (PAO1260) was constructed which carried both sex factors by taking PAO1231 (which is Leu$^+$, FP39$^+$ and mercury-sensitive) and infecting it with FP2 from PTO13. The presence of FP2 in PAO1231 could be detected by the acquisition of mercury resistance. The stability of this strain for each sex factor could be readily tested by the stability of its response to mercury and the ability to grow on minimal medium. If FP2 was lost the clone would become mercury-sensitive. If FP39 was lost, the determinant for leucine biosynthesis would be lost and such a clone would become leucine requiring.

When PAO1260 was grown in broth overnight from a small inoculum (12–15 generations) then plated out overnight on MIA to give isolated single colonies (25–30 generations), it was found that 0·3 % of such colonies tested had lost FP2, 5% had lost FP 39 and $< 0{\cdot}1\%$ had lost both. Controls of each sex factor on its own (using strains PAO1231 and PTO13) did not exhibit any spontaneous loss of sex factor during the same number of generations. During the overnight growth in broth, the cell densities achieved ($> 5 \times 10^8$ cells/ml) probably allowed some reinfection by both sex factors, hence it is likely that the actual losses of each sex factor may be even higher than those observed. In view of the observed variability of infectivity of PAO male strains by sex factors, precise measurement of this aspect of the instability of each sex factor is difficult. No instability of FP2 or FP39 when carried alone in PAO strains has ever been observed and hence this degree of loss of each sex factor when carried together is significant evidence that they are incompatible and hence presumably related.

The two sex factors did not act entirely independently of one another with respect to transferability when both were present in the same cell; FP2 was more efficiently transferred than FP39 in such a situation. The strain PAO1235 (FP2$^+$ FP39$^+$) containing both sex factors and constructed by the same procedure used for PAO1260 was mixed with PAO361 (*leu-38*, FP$^-$) for 6 h in broth at 37 °C, the ratio of donor:recipient cells being 100:1. This ratio was necessary in order to get adequate sex-factor transfer over this period of time. The transfer of FP39 was detected selecting Leu$^+$ clones and transfer of FP2 selecting for mercury-resistant clones. After 6 h of mating it was found that 10 % of the recipient population received FP2, while only about 1 in 10^5 of the recipients received FP39. It can be concluded that in a strain such as PAO1235 (FP2$^+$ FP39$^+$), the ability to transfer FP2 remains near normal, whilst the ability to transfer FP39 is greatly impaired.

4. DISCUSSION

Two current problems in the genetic study of *P. aeruginosa* are those of chromosomal mapping and the mechanism of chromosome transfer by sex factors. FP2 apparently promotes chromosome transfer predominantly from one site and this prevents mapping of all regions of the chromosome. The purpose of the current study was to isolate sex factors promoting transfer from sites other than that

occurring with FP2. Sex factors were readily found amongst wild-type strains of *P. aeruginosa* and one, FP39, was examined in some detail.

FP2 and FP39 differed in their abilities to confer mercury resistance, the ability to carry genetic information for leucine synthesis and in the times at which they promoted entry of a range of chromosome markers during conjugation. Preliminary experiments on exclusion and incompatibility suggest that there may be some relationship between these two sex factors.

The nature of the genetic determinant of leucine synthesis carried by FP39 is unknown. In the presence of FP39, strains possessing a mutation in a particular gene determining leucine biosynthesis acquired the ability to grow on minimal medium. This was not restricted to one particular mutation at this locus. Three independently isolated mutants of this particular leucine marker (*leu-38*, *leu-8*, *leu-41*) showed the same response to the presence of FP39. The determinant could be a structural gene for an enzyme involved in leucine biosynthesis for which these strains are deficient. The fact that three independently isolated mutants of this one leucine locus showed the same effect and these mutations were isolated using different mutagens (manganese chloride, ethyl methane sulphonate and nitrosoguanidine) suggests that it is not an informational suppressor but is more likely to be a duplication of the leucine gene carried by the sex factor. A similar case, although not involving a plasmid, has been reported in *Salmonella typhimurium* by Kemper & Margolin (1969). They showed that a suppressor gene, *supQ*, was specific for *leuD* gene mutations and could act to suppress even a deletion of the *leuD* region, showing that *supQ* was acting as a substitute gene and not in the usual sense of a translational suppressor,

There are other possibilities to explain the nature of the Leu$^+$ determinant carried by this sex factor. For example, FP39 may be a merogenote carrying the *leu-38*$^+$ gene on a chromosomal piece such that it is unlinked to other genes to which *leu-38* is linked on the chromosome. Alternatively, FP39 could be carrying a duplication of some chromosomal region containing a suppressor for the *leu-38* gene. This system may have possibilities for the development of a stable partial diploid structure, which so far has not been achieved for *P. aeruginosa*.

The phenotypic characteristics of FP39 and its relationship to FP2 are related to the question of the spatial situation of sex plasmids in *P. aeruginosa*. As yet there is no evidence that they are integrated into the chromosome. As suggested by Stanisich & Holloway (1969*a*), it is possible that transfer of chromosome and transfer of sex factor in conjugation may be independent procedures, indicating either that sex factors are not integrated into the chromosome or, in view of the likelihood of there being more than one copy of the sex factor per cell (Stanisich, 1972), that *P. aeruginosa* sex factors may occur in the one cell in both the integrated and extrachromosomal states.

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