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Pleiotropy of *p*-fluorophenylalanine-resistant and antibiotic hypersensitive mutants of *Pseudomonas aeruginosa*

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SUMMARY

Mutants of *Pseudomonas aeruginosa* isolated as being resistant to *p*-fluorophenylalanine (FPA-r) are pleiotropic, showing patterns of phenotypic alteration of host-controlled modification, growth rate at 37 and 43 °C, ability to grow on minimal medium, response to antibiotics and ribosomal characteristics. Alterations in host-controlled modification are also found in FPA-r mutants of *Escherichia coli* B.

The pleiotropy arising in these FPA-r mutants of *P. aeruginosa* appears to result from alterations in the specificity of protein synthesis. Phenotypic variations in host-controlled modification of the type found in the FPA-r mutants may provide a method for the detection of suppressor mutations which act by miscoding.

1. INTRODUCTION

Host-controlled modification (HCM) in *Pseudomonas aeruginosa* has been shown to be under a variety of genetic controls (Rolfe & Holloway, 1968; Holloway, 1969). The demonstration that over 50% of mutants of *P. aeruginosa* resistant to *p*-fluorophenylalanine (FPA) showed concomitant alteration in HCM cannot be readily explained by the usual mechanisms associated with amino acid analogue resistance including (i) cellular impermeability to the analogue or (ii) alterations in the regulation of biosynthesis of the amino acid with which the analogue competes. However, in the case of FPA, Fangman & Neidhardt (1964) have shown that in one FPA-r mutant of *E. coli* there was an alteration of an altered amino acyl transfer ribonucleic acid synthetase. This suggested a relationship between FPA resistance and protein translation mechanisms. Waltho & Holloway (1966) and Waltho (1968) demonstrated that the basis of FPA resistance in a class of spontaneous FPA-r mutants of *P. aeruginosa* strain PAT could not be explained by any of the previously characterized mechanisms, and that for some of these mutants the FPA-r phenotype was suppressed by mutation to streptomycin resistance. The present paper reports an investigation of the relationship between FPA-r mutations and phenotypic changes in HCM and other properties in *P. aeruginosa* strain PAO.

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2. MATERIALS AND METHODS

The bacterial strains used were as follows:

Strain no. (*P. aeruginosa*)	Genotype	HCM phenotype
PAO 1	Prototroph, FP –	$R^+_{PAO}M^+_{PAO}$
PAO 174	Prototroph, *str*-2 FP –	$R^+_{PAO}M^+_{PAO}$
PAO 67	*his*-67, *ese*-2, FP –	$R^+_{PAO}M^+_{PAO}$
PAO 170	*leu-38*, FP +	$R^+_{PAO}M^+_{PAO}$
PAO 200	*leu-38*, *ahs-1*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 181	*leu-38*, *fpa-6*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 182	*leu-38*, *fpa-5*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 205	*leu-38*, *fpa-7*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 206	*leu-38*, *fpa-8*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 207	*leu-38*, *fpa-9*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 208	*leu-38*, *fpa-10*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 210	*leu-38*, *fpa-11*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 211	*leu-38*, *fpa-12*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 214	*leu-38*, *fpa-13*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 215	*leu-38*, *fpa-14*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 216	*leu-38*, *fpa-15*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 1161	*leu-38*, *rmo-10*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 1162	*leu-38*, *rmo-11*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 1163	*leu-38*, *rmo-12*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 1164	*leu-38*, *rmo-13*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 1165	*leu-38*, *rmo-14*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 1166	*leu-38*, *rmo-15*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PAO 1168	*leu-38*, *rmo-16*, FP 2 +	$R^-_{PAO}M^+_{PAO}$
PAO 1169	*leu-38*, *rmo-17*, FP 2 +	$R^-_{PAO}M^-_{PAO}$
PTS 271	Prototroph, strain 271 FP –	$R^+_{PTS}M^+_{PTS}$
E. coli B 707	*gal*$^-$ *met*$^-$	$R^+_BM^+_B$
E. coli K 12 C 600	Prototroph	$R^+_KM^+_K$

All mutants carrying *leu-38* were derived from PAO 170. The *E. coli* strains were kindly provided by Dr W. Wood.

Abbreviations. Genotypes: *ahs*, antibiotic hypersensitive; *fpa*, fluorophenylalanine resistance; *gal*, galactose; *leu*, leucine; *met*, methionine; *rmo*, restriction and modification; *str*, streptomycin. Phenotypes: R, restriction; M, modification; PAO, strain 1 (PAO) specificity; PTS, strain 271 (PTS) specificity; B, strain B specificity; K, strain K specificity.

Both genotypes and phenotypes are listed above, but in the text it is more convenient to refer to phenotypes because of the pleiotropic nature of some of the mutations involved.

Phage used in this study were B3c (Holloway, Egan & Monk, 1960; Holloway, 1965), the transducing phage G 101 (Holloway & van de Putte, 1968), D 3 (Holloway *et al.* 1960) and the *E. coli* phage λc (kindly provided by Dr W. Wood).

General culture procedures. Media, maintenance of stock cultures and growth conditions were the same as previously described by Holloway (1965) and Stanisich

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& Holloway (1969). Cells were disintegrated for *in vitro* work using a French Press and following the procedures of Isaac & Holloway (1968). All mutants were isolated following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) (Fargie & Holloway, 1965). The screening method for characterizing restriction and modification phenotypes, the isolation of streptomycin-resistant mutants and the isolation of FPA-r mutants were as previously reported by Rolfe & Holloway (1968). Antibiotic-hypersensitive mutants (mutants with an increased sensitivity to particular antibiotics) were obtained by replica plating single clones from a mutated population on to nutrient agar (NA) plates supplemented with either 15 μg/ml streptomycin, 100 μg/ml erythromycin or 50 μg/ml spectinomycin. Bacterial growth rates were determined in Difco Heart Infusion Broth (HIB) supplemented with 0·1 % Tween 80, the addition of Tween 80 reducing the clumping of cells to allow more reproducible growth curve estimations. Transduction procedures were similar to those used by Fargie & Holloway (1965).

Modification properties were determined using the phage plating method of Rolfe & Holloway (1968) and also by conjugation to both R^{+}_{PAO} and R^{-}_{PAO} strain 1 recipients (PAO 67 grown at 37 °C and 43 °C respectively; Holloway, 1965).

In vitro *ribosome studies.* The incorporation of [^{14}C]phenylalanine (phe) into poly-phe was measured using ribosomes isolated from *P. aeruginosa*. Polyuridylic acid was used as the template together with *E. coli* B phe t-RNA. To facilitate the *in vitro* protein synthesis, a crude enzyme fraction was isolated from *E. coli* B. The fraction contained the T fraction (binding of GTP to aminoacyl t-RNA and transfer to ribosomes), and the G fraction (translocation of peptidyl t-RNA and release of inorganic phosphate from GTP; Lipmann, 1969). Collectively these two fractions are called the TG fraction. The *E. coli* B t-RNA and TG fraction were shown to be functionally interchangeable for ribosome preparations from either *E. coli* B or *P. aeruginosa.*

Preparation of ribosomes from P. aeruginosa *and* E. coli. The method for the preparation and purification involved the commonly used differential centrifugation technique (Apirion, 1966). Ribosomal concentrations were estimated at 260 mμ assuming that an O.D. of 1·0 corresponds to approximately 60 μg ribosomes/ml. The purified ribosomes were stored frozen at −20 °C in 0·01 M tris-HCl, pH 7·5, buffer containing 0·01 M-Mg acetate.

Preparation of TG fraction from E. coli *B.* The procedure for removal of ribosomes followed by ammonium sulphate fractionation of the supernatant was essentially that used by Nishizuka & Lipmann (1966).

Activation of t-RNA with ^{14}C-phe. This method was similar to that described by von Ehrenstein & Lipmann (1961).

Ribosomal assay. The assay method was similar to that described by Nishizuka & Lipmann (1966). The incorporation mixture contained the following: 40 mM tris-HCl, pH 7·5; 80 mM-KCl; 5 mM 2-mercaptoethanol; 0·2 mM GTP; 9 mM-$MgCl_2$; 72 μg TG; 4 μg polyuridylic acid and 0·02 ml of ^{14}C-phe t-RNA which contained approximately 9000 c.p.m. and approximately 10–15 μg ribosomes. The

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final volume was adjusted to 0·5 ml with distilled water. All determinations were carried out in duplicate. Controls included duplicate determinations in the absence of (i) polyuridylic acid, (ii) TG and (iii) ribosomes. The assay tubes were incubated for 15 min at 37 °C, then the reaction stopped by the addition of 3 ml of 7 % TCA. The mixture was heated at 85–90 °C for 15 min, cooled on ice, then filtered through a Millipore filter and washed three times with 2 ml of 7 % TCA. The filters were dried in scintillation bottles (30 min at 110 °C) then counted in an aqueous scintillator (Bruno & Christian, 1961).

3. RESULTS

(i) *Pleiotropy in FPA-r mutants*

Rolfe & Holloway (1968) showed that over 50 % of FPA-r mutants of *P. aeruginosa* PAO 1 showed alteration in HCM properties. We have examined FPA-r mutants of another strain of *P. aeruginosa*, PTS 271, and of *E. coli* B, with essentially similar results. Thirty-three per cent of the FPA-r mutants of PTS 271 showed alterations in restriction when tested against B3c.1. Forty-four per cent of FPA-r mutants of *E. coli* B showed changes in restriction properties, this being tested by cross streaking against λc propagated on *E. coli* K 12, phage of this modification type being normally restricted by *E. coli* B (Wood, 1966). This last result is important in demonstrating that this aspect of pleiotropy of FPA-r mutants is likely to be of a more general nature rather than merely being peculiar to some strains of *P. aeruginosa*.

It is difficult to see how such alterations in HCM could be related to permeability to FPA, or to the regulation of phenylalanine biosynthesis. It is more likely that resistance to FPA is one expression of a mutation affecting some more fundamental aspect of cellular metabolism such as protein synthesis. If this were so, other phenotypic effects could be expected in FPA-r mutants. A number of characteristics of such mutants have been examined and it has now been found that FPA-r mutants are pleiotropic for a range of properties.

(*a*) *Growth rate*

When selection is made for FPA-resistant colonies on minimal medium containing 1 mg/ml of FPA, there is a marked variation in colony size, suggesting that different mutants have different growth rates. This was confirmed by measuring the growth rate in nutrient broth and the generation time of different clones was found to vary from 33 min to over 60 min. Furthermore, it was shown that only 2 % of the faster-growing mutants (33–40 min generation time) also showed changes in HCM, while over 34 % of the slower-growing mutants (generation time greater than 40 min) showed such changes. It was also found that 45 % of the FPA-r mutants did not grow at 43 °C and that this group of mutants had generation times greater than 40 min when grown at 37 °C. These results suggest that a spectrum of mutants is being isolated with varying degrees of disturbance of some essential

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cellular function which is reflected by the isolation of mutants with a range of pleiotropic characteristics.

(*b*) *Auxotrophy*

A convenient test to examine the suggestion that a variety of functions can be potentially affected following mutation to FPA-r is to isolate FPA-r mutants under conditions where a variety of biosynthetic functions could be affected without being lethal. Under the usual procedure for the isolation of FPA-r mutants, plates containing only FPA and the particular supplement required for the parent strain are used, so that if any change in the phenotype to auxotrophy resulting from the

Table 1. *Auxotrophic requirements induced following mutation to FPA-r and the analysis of spontaneous and transduction revertants to prototrophy*

		Response to FPA of:	
Mutant no.	Auxotrophic requirement	Spontaneous revertants	Transductional revertants
1	Leucine	—	FPA-s
2	Methionine	FPA-s	—
3	Isoleucine + valine	—	FPA-s
4	Methionine	—	FPA-s
5	Not determined*	—	FPA-s
6	Tryptophan	FPA-s	—
7	Cysteine	FPA-s	—
8	Not determined*	FPA-s	—
9	Leucine	FPA-s	—

* Presumably multiple auxotrophic requirements.

mutation to FPA-r should have arisen, it would not have been detected by the screening procedure. To overcome this, an NG mutated population of PAO 1 was plated on to the standard FPA selection plates and also plated on to minimal plates supplemented with a mixture of arginine, aspartic acid, asparagine, adenine, cysteine, glutamate, histidine, iso-leucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine, uracil and valine, each at a concentration of 100 μg/ml and FPA (1 mg/ml). The standard selection procedure yielded $1{\cdot}6 \times 10^3$ FPA-r clones/10^8 cells whereas the amino acid supplemented plates yielded $1{\cdot}3 \times 10^4$ resistant clones/10^8 cells in the mutated population. When clones from the amino acid supplemented plates were purified on NA plates and further tested it was found that some did not grow on minimal medium. Nine of these auxotrophic FPA-r mutants were selected and their growth requirements determined. As shown in Table 1, the mutants had a variety of auxotrophic requirements. Furthermore, prototrophic derivatives of these mutants could be isolated either by plating 10^9–10^{10} cells of a mutant on minimal medium or by transduction using phage G 101 grown on the prototrophic donor strain PAO 1. Such prototrophic derivatives also became sensitive to FPA, although the spontaneous revertants showed some variation in the degree of sensitivity acquired.

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(c) *Relationship between FPA-r, antibiotic hypersensitivity and HCM*

The above results provide support for the view that the mutations which result in FPA resistance affect other aspects of cellular function, most likely those involved in translational mechanisms. It is known that certain antibiotics act at the translational level of protein synthesis and that mutants with altered ribosomes can show a hypersensitivity to certain antibiotics (Apirion, 1967). A relationship between FPA-r mutants and this type of change has been sought and it has been found that FPA-r mutants may also show increased sensitivity to antibiotics.

Table 2. *Antibiotic hypersensitivity patterns of 300 FPA-r mutants of PAO 170*

Phenotypic classes	No. of mutants
SM-hs	22
ER-hs	8
SM-hs, ER-hs	19
Wild-type response	251

hs indicates hypersensitivity to the respective antibiotics.

Table 3. *Antibiotic hypersensitivity patterns and growth rates of FPA-r R^- mutants of PAO 170*

Mutant	Antibiotic hypersensitivity pattern	Restriction phenotype	Generation time at 37 °C (min)
PAO 170 (parent)	Normal	$R^+_{PAO}M^+_{PAO}$	37
PAO 181	Normal	$R^-_{PAO}M^-_{PAO}$	37
PAO 206	Normal	$R^-_{PAO}M^-_{PAO}$	37
PAO 214	Normal	$R^-_{PAO}M^+_{PAO}$	38
PAO 205	SM-hs	$R^-_{PAO}M^-_{PAO}$	60
PAO 207	SM-hs	$R^-_{PAO}M^-_{PAO}$	48
PAO 208	SM-hs	$R^-_{PAO}M^+_{PAO}$	60
PAO 210	SM-hs	$R^-_{PAO}M^+_{PAO}$	52
PAO 215	SM-hs	$R^-_{PAO}M^+_{PAO}$	54
PAO 216	SM-hs	$R^-_{PAO}M^-_{PAO}$	47
PAO 182	SM-hs, ER-hs	$R^-_{PAO}M^-_{PAO}$	90
PAO 211	SM-hs, ER-hs	$R^-_{PAO}M^+_{PAO}$	65

The antibiotics used were streptomycin (SM), erythromycin (ER) and spectinomycin (SP). The minimal concentration of each of these antibiotics to produce growth inhibition of PAO 170 (*leu-38*, FP+) was 15 μg/ml for SM, 100 μg/ml for ER and 50 μg/ml for SP and antibiotic hypersensitivity was assessed using these concentrations. Three hundred FPA-r mutants of PAO 170 were selected, purified by single colony isolation and their antibiotic hypersensitivity patterns determined (Table 2).

It is seen that 16% of FPA-r mutants were also hypersensitive to either or both SM and ER. None of the mutants were hypersensitive to SP. A similar experiment

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to determine the percentage of antibiotic hypersensitive mutants occurring in a similarly mutated population of PAO 170 but without selection for FPA-r showed that they occurred at a frequency of 0·8 %. Thus not only is an unusually high proportion of FPA-r mutants also antibiotic hypersensitive, but such a proportion cannot be explained by double mutation. As suggested above, it appears that there is a range of FPA-r mutants with varying degrees of metabolic disturbance. It is likely that the more extreme the disturbance the greater the range of phenotypic characteristics showing other than wild-type expression. This view is supported by the finding that FPA-r mutants which are R^-_{PAO} are more commonly antibiotic-sensitive than a random sample of FPA-r mutants and such mutants characteristically have longer generation times. Table 3 shows that 8 of the 11 FPA-r R^- mutants were also antibiotic hypersensitive and that the antibiotic primarily

Table 4. *Classes of antibiotic hypersensitive mutants isolated from PAO 170 and their HCM properties*

Mutant classes	No. isolated	No. of each class with restriction properties		
		R^+_{PAO}	R'_{PAO}*	R^-_{PAO}
SM-hs	9	6	3	0
ER-hs	21	13	8	0
SP-hs	1	0	1	0
SM-hs, ER-hs	12	7	3	2
SM-hs, ER-hs, SP-hs	14	9	5	0
Total	57	35	20	2

* R′, Partial restriction.

concerned with the hypersensitivity was streptomycin. If changes in antibiotic sensitivity and HCM response are a reflexion of translational infidelity then it follows that similar results to those of Table 3 should be found if mutants are selected as being antibiotic-hypersensitive and then tested for HCM characteristics. This has been done and the results are shown in Table 4.

It is seen that there is a high correlation between changes in antibiotic hypersensitivity and changes in restriction and it is perhaps to be expected that most of the mutants examined showed a partial rather than a complete loss of restriction properties. All the mutants shown in Table 4 were found to be FPA-s like the parent from which they were derived.

(ii) *Effect of mutation to SM-r on the FPA-r Res− mutants*

Mutations to streptomycin resistance have been shown to act at the ribosomal level of translation (Davies, 1966). Mutations to streptomycin resistance (SM-r) in wild-type PAO 1 produce no significant alterations in HCM properties (Rolfe & Holloway, 1968) although changes in HCM properties have been reported to occur in SM-r mutants of *E. coli* (Lederberg, 1957; Dussoix & Arber, 1962). Suppression of FPA-r following mutation to SM-r has been reported by Waltho & Holloway

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(1966) and suppression by streptomycin resistance of both FPA-r and HCM phenotype would be further evidence of a close cellular relationship for these two characteristics. Four of the FPA-r R^- mutants (PAO 205, 206, 207 and 210) were mutated to SM-r then their resistance to FPA was determined and their restriction properties studied. The proportion of mutants that had acquired a dependence to this antibiotic was also determined. Similar results were obtained for all these mutants and results for PAO 205 given in Table 5 are typical of PAO 206, PAO 207 and PAO 210.

Table 5. *Effect of mutation to SM-r on the expression of FPA-r and HCM in PAO 205* ($R^-_{PAO}M^-_{PAO}$)

SM	FPA	Phenotype restriction	Occurrence (%)
SM-r	FPA-r	R^-_{PAO}	16
SM-r	FPA-s	R^-_{PAO}	4
SM-r	FPA-s	R'_{PAO}	2
SM-r	FPA-s	R'_{PAO}	4
SM-r	FPA-s	R^-_{PAO}	0
SM-d	FPA-s	R^-_{PAO}	27
SM-d	FPA-s	R'_{PAO}	12
SM-d	FPA-s	R'_{PAO}	35
SM-d	FPA-s	R^-_{PAO}	0

R'_{PAO}, Partial restriction; SM-d, streptomycin-dependent: the frequency of occurrence of SM-d mutants among SM-r mutants selected at random following the mutation of PAO 170 was 10% (unpublished results). The SM-r mutants were selected, after NG treatment, on 250 μg/ml SM-NA plates. These mutants were purified on SM-NA plates. FPA-r was tested on minimal plates supplemented with leucine, FPA (1 mg/ml) and SM (250 μg/ml). Phage plating properties were determined by cross-streaking against each phage on 250 μg/ml SM-NA plates. All mutants tested grew on minimal plates supplemented with leucine and SM (250 μg/ml).

The results in Table 5 illustrate that selection of SM-r mutants from PAO 205 resulted in suppression of both the FPA-r and restriction deficient phenotypes in many of the mutants. Several SM-r mutants were found to form plaques with a reduced efficiency for both B3c.271 and B3c.1 and appear to have suffered a non-specific effect which influences phage maturation generally. The observation that a high proportion of the SM-r mutants were in fact SM-d was unexpected. When selection is made for SM-r and SM-d mutations in an FPA-s strain, SM-r mutants are isolated much more frequently than SM-d mutants. These results suggested an involvement of the ribosomes themselves in the phenotypic expression of FPA-r mutations, perhaps due to the presence of defective proteins in the ribosomes. This was experimentally tested by an examination of certain ribosomal properties.

(iii) *Ribosomal function in* Pseudomonas aeruginosa

Streptomycin sensitivity and heat-stability were determined *in vitro* using ribosomes isolated from the parent strain, PAO 170. To determine whether differences in properties of ribosomes from the FPA-r, R^- mutants could be detected, the

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streptomycin sensitivity and heat-stability patterns were estimated and compared to those of ribosomes isolated from PAO 170. Ribosomes isolated from a SM-r mutant (PAO 174) and a SM-hs mutant (PAO 200) were included for comparison and the results are given in Table 6.

Table 6. In vitro *sensitivity to streptomycin and heat-stability of ribosomes isolated from different classes of* Pseudomonas aeruginosa *mutants*

Ribosomal origin	Phenotype	[^{14}C]phenylalanine incorporation activity remaining (%) at streptomycin concentrations (μg/0·5 ml)		
		0	91	187
PAO 170	SM-normal	100	51	37
PAO 174	SM-r	100	83	70
PAO 200	SM-hs, R^-_{PAO}	100	88	88
PAO 206	FPA-r, R^-_{PAO}, SM normal	100	58	43
PAO 207	FPA-r, R^-_{PAO}, SM-hs	100	52	37
PAO 210	FPA-r, R^-_{PAO}, SM-hs	100	62	49
		At pre-incubation temperature (°C)		
		37°	47°	50°
PAO 170	—	100	31	18
PAO 174	—	100	31	18
PAO 200	—	100	31	21
PAO 206	—	100	55	49
PAO 207	—	100	36	28
PAO 210	—	100	30	31

For streptomycin-sensitivity determinations the antibiotic was added prior to incubation. For heat-stability determinations ribosome preparations were pre-incubated at a concentration of 200 μg/ml at the stated temperatures for 30 min, then 0·05 ml samples were withdrawn and assayed.

It is seen that the ribosomes from streptomycin-resistant mutants show a higher *in vitro* resistance to SM. Surprisingly, so do the SM-hs strains. The ribosomes from the FPA-r strains tested (PAO 206 and PAO 210) show some increase in resistance to SM as compared to the wild type whether or not they were originally SM-hs or normal in their response to SM.

In vitro heat-stability properties illustrate that ribosomes from PAO 206 differ significantly from the control. The differences from the control values for ribosomes from PAO 207 and PAO 210 were not large enough at both temperatures to state definitely whether they are significant. It can, however, be concluded that some mutants selected as being FPA-r and R^- do carry demonstrable differences in ribosomal properties as detected by *in vitro* tests.

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(iv) *Study of directly isolated mutants carrying changes in HCM properties*

It has been previously reported that mutants with altered restriction and modification properties can be readily isolated by directly testing survivors of mutagenesis for their restriction and modification properties in *P. aeruginosa* (Rolfe & Holloway, 1968). Mutants having a range of phenotypes including $R^{-}_{PAO}M^{+}_{PAO}$, $R'_{PAO}M^{+}_{PAO}$, $R^{-}_{PAO}M'_{PAO}$ and $R^{-}_{PAO}M^{-}_{PAO}$ were characterized.

We have now examined mutants isolated in this way for pleiotropic characters such as are found with the FPA-r mutants which show variations in HCM properties.

Eight R^{-} mutants (PAO 1161, 1162, 1163, 1164, 1165, 1166, 1168 and 1169) were isolated by direct selection following NG treatment of PAO 170. A comparison was carried out between these mutants and R mutants following selection of FPA-r. By contrast to the FPA-r R^{-} mutants, the cell properties of these mutants were considerably different. These differences were (i) the growth rates were all similar to the parent (PAO 170) and ranged from 32 to 36 min, (ii) none of these mutants were antibiotic-hypersensitive, (iii) little, if any, phenotypic suppression in HCM properties was observed following mutation to SM-r. As found with the FPA-r R^{-} mutants, approximately half of this type of mutant was also M^{-}. It is thus seen that mutants selected on the basis of having altered HCM properties do not show the pleiotropy of FPA-r mutants and hence it can be concluded that the former are more likely to represent changes in enzymes directly concerned with restriction and modification, rather than through some more indirect mechanism.

4. DISCUSSION

This paper not only confirms the previously described relationship between FPA-r and HCM (Rolfe & Holloway, 1968) but shows that a wider range of phenotypic changes commonly occurs in FPA-r mutants of *P. aeruginosa*. These include a slower rate of growth at 37 °C, ability to grow at 43 °C, antibiotic-hypersensitivity and auxotrophy. The results obtained with reversion of FPA-r auxotrophs and suppression by mutation to SM-r, together with the high degree of correlation between FPA-r mutation and alterations in other phenotypic characteristics strongly point to the cause of these pleiotropic effects as being a single mutation. The observed pleiotropy confirms the earlier suggestion that the effect of FPA-r mutations on HCM is indirect, and in contrast to those HCM mutations isolated by selecting only for alterations in restriction phenotype (Rolfe & Holloway, 1968). These latter presumably arise through mutations affecting the structural genes of restriction and modification.

The pleiotropic effect of FPA-r mutants appear to arise because the primary mutation affects the mechanism of protein synthesis. The mutants presumably become resistant to the lethal effects of FPA because either FPA is not activated or if activated it cannot be incorporated into protein. Hence FPA-r mutations represent changes in either t-RNA genes or, less likely, t-RNA amino acyl

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synthetase genes and result in miscoding during protein synthesis which results in proteins with other than wild-type function.

It is not clear whether such miscoding results in only altered protein with defective function or, through a degree of ambiguity in coding, there is a mixture of normal and altered protein. In the latter case variations in the proportions of the two types of protein could result in various degrees of pleiotropy, the more extreme mutants having a greater proportion of defective protein.

We are inclined to the view that mutations in genes producing t-RNA molecules could account for all the observed pleiotropic effects. For example, a mutation could occur in a phenylalanine t-RNA such that it would no longer be able to activate FPA. If, in addition, the specificity of anticodon recognition became altered, the strain carrying this mutation would be both FPA resistant and demonstrate a lack of fidelity in protein synthesis. However, such changes need not be restricted to the phenylalanine t-RNA and perhaps a mutation in another t-RNA species could result in successful competition with phenylalanine t-RNA for the phenylalanine codon, together with a loss of activity for the homologous codon. This mutated t-RNA would not have the property of activating FPA and hence the strain carrying it would show higher resistance to the analogue. Auxotrophy of such an FPA-r mutant could result from the inefficiency of the minor species t-RNA of the amino acid corresponding to the mutated t-RNA, such that activation would only take place in the presence of higher concentrations of the particular amino acid. It is thus possible that in view of the variety of growth requirements found in these auxotrophs the FPA-r mutants isolated are genetically heterogeneous.

The effect of SM-r mutations on expression of FPA-r mutations is comparable to other bacterial systems where it has been shown that mutation to SM-r can reduce or eliminate the effect of a pre-existing suppressor gene (Lederberg, Cavalli-Sforza & Lederberg, 1964; Davies, 1966; Gartner & Orias, 1966; Kuwano, Ishikawa & Endo, 1968; Otsuji & Aono, 1968). Indeed the properties of FPA-r mutants are very similar to either missense or ochre suppressor mutations, one ochre suppressor mutant of *E. coli* having properties very similar to those of FPA-r mutants in *P. aeruginosa*, namely slow growth rate, streptomycin-hypersensitivity and ribosomal defects *in vitro* (Gartner *et al.* 1969).

It is possible but less likely that FPA-r mutants studied in this paper represent changes in t-RNA amino acyl synthetase enzymes similar to the mutant of *E. coli* described by Fangman & Neidhardt (1964). Changes at this level of the protein synthesis mechanism could result in both FPA resistance and pleiotropy due to lack of specificity in an altered t-RNA synthetase. This could result in other amino acids being activated in place of phenylalanine to produce the observed pleiotropy. Furthermore, if this is the only possible mutational change able to produce the observed effects, only one gene would be involved so that it is important that mapping of the loci producing FPA resistance be carried out. Attempts to do this by conjugation have not been successful as the FPA-r mutants appear to map into that region of the chromosome between the insertion of the FP2 sex factor

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and the *leu-38* marker. This region cannot be reliably mapped at present owing to the distribution of sites of origin of the known sex factors of *P. aeruginosa* (Holloway, Krishnapillai & Stanisich, 1971).

The properties of ribosomes from *P. aeruginosa* FPA-r mutations do not differ greatly from those of the wild-type bacteria and we favour the view that these changes are secondary, resulting from alterations in the ribosomal protein due to miscoding.

The involvement of these FPA-r mutants in mechanisms of protein synthesis was first recognized by their effect on HCM. HCM is evidently not an essential cell function and changes in restriction and modification are not expected to affect cell viability. The role of the FPA-r mutations here is that of a suppressor mutation, with the difference that the observed change in phenotype due to the suppressor is from the wild-type to the mutant phenotype. The correlation with changes in HCM may be a useful test for detecting mutational changes in coding fidelity as the screening of large numbers of clones can be readily undertaken. Perhaps the detection of a wider range of cellular functions affected by FPA-r mutations could be accomplished by the use of temperature-sensitive FPA-r mutations, although preliminary attempts to find such mutants have not been successful.

Preliminary attempts were made to find suppressor-sensitive mutants of bacteriophage by mutating phage D 3 (Holloway *et al.* 1960) and looking for mutants which would propagate on FPA-r mutants (as the equivalent of SU^+ strains) and not on wild-type strains. A variety of such mutants were found which plated with a reduced efficiency of plating varying from 5- to 100-fold on an FPA-s strain when compared to the FPA-r. While it has not yet been possible to map or show complementation of such mutants, the isolation of such mutants is additional evidence to support our views on the nature of the mutants (Holloway, Jennings & Dunn, unpublished results).

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