Genetical and Biochemical Aspects of Resistance to *p*-Fluorophenylalanine in *Saccharomyces cerevisiae*

By P. M. RHODES AND D. WILKIE

*Department of Botany and Microbiology, University
College London, London WC1E 6BT*

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SUMMARY

Growth of haploid yeast strains was inhibited by the phenylalanine (PA) analogue DL-*p*-fluorophenylalanine (FPA) in yeast extract media containing 0.2 mg PA/ml. Most strains had a maximum FPA tolerance of about 0.25 mg/ml when glycerol was the carbon source and 0.5 mg/ml in glucose medium. Spontaneous FPA-resistant mutants isolated on glucose medium showed little or no increase in FPA tolerance over that of the parent when metabolizing glycerol. Resistance was controlled by a different nuclear gene in each of four mutants analysed. In a proportion of the mutants the amount of FPA incorporated into cellular proteins in competition with PA was less than into the proteins of sensitive parental cells, whether glucose or glycerol was used as carbon source. This suggests that the mutational change allowed the cytoplasmic system to discriminate against the analogue without affecting its incorporation into mitochondrially-synthesized proteins. Although attempts to measure the latter were not made, the observed decrease in respiratory activity of cells grown in the presence of FPA suggests such incorporation. In other mutants showing resistance to FPA in glucose medium, the amount of FPA incorporated into cellular proteins varied with the carbon source, less analogue being incorporated in glucose medium than in glycerol medium.

INTRODUCTION

The discovery of tRNA species and aminoacyl-tRNA synthetases intrinsic to mitochondria of *Neurospora*, rat liver, *Tetrahymena* and yeast (see Borst, 1972) suggested the possibility of distinguishing between the respective tRNA systems of cytoplasm and mitochondrion by differential response to inhibitors (Wilkie, 1970). Inhibitors known to act at the metabolic level of aminoacyl-tRNA formation are the structural analogues of the amino acids. Incorporation of analogues in place of natural amino acids generally stops growth since analogue-containing proteins are usually defective (see review of Fowden, Lewis & Tristram, 1967). In micro-organisms sensitive to particular analogues, resistant mutants have been isolated in which the mechanism of resistance lies in an altered aminoacyl-tRNA synthetase which charges tRNA with analogue at a much lower rate than with the corresponding amino acid. In eukaryotic cells it is theoretically possible to pick up similar mutational changes in one or other of the two tRNA systems, cytoplasmic or mitochondrial, but a first requirement is to demonstrate inherent differences in the two systems. This is possible in the facultative anaerobe *Saccharomyces cerevisiae* since an inhibitory effect on the mitochondria does not prevent growth provided fermentable substrate is available, although it would do so with a non-fermentable carbon source. Thus, a situation in which the mitochondrial system is more sensitive to the presence of

an analogue than the cytoplasmic system can readily be identified in yeast cells. It should be possible to exploit this situation to obtain analogue-resistant mutants of both the cytoplasmic and mitochondrial tRNA systems.

In the experiments reported here, the relative sensitivities of the cytoplasmic and mitochondrial tRNA systems in *S. cerevisiae* to the phenylalanine (PA) analogue DL-*p*-fluorophenylalanine (FPA) has been studied.

METHODS

Cultural conditions. Yeast extract (0.5 %, w/v), with 2 % (w/v) glucose (YED) or with 4 % (v/v) glycerol (YEG), was used as the fermentable or non-fermentable medium respectively. These yeast extract media contained 0.2 mg PA/ml, according to the manufacturer's (Difco) estimate. Standard conditions of culture, crossing, tetrad analysis and random ascospore analysis were employed (Mortimer & Hawthorne, 1969). Haploid strains 10, 26 and 27 of this laboratory were used, carrying the nuclear markers α , *arg*, *met*; *a*, *ade-2*; and *a*, *ura*, *his*, respectively.

Tests of inhibition. FPA was added to agar medium at concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 7.5 mg/ml. Tolerance level is defined as the highest analogue concentration still permitting growth. Methods of isolation and classification of resistant mutants, and the production and testing of diploids were as described in Wilkie (1970). Cell density (absorbance) of shake cultures was measured in an EEL colorimeter using a red filter (λ_{max} 607 nm). Absorption spectra of whole cells were obtained in a Unicam SP800 spectrophotometer using high cell density ($\geq 10^8$ /ml).

Amino acid incorporation into protein. Strains were grown to exponential phase in YED, harvested by centrifugation and resuspended at room temperature in fresh YED medium to give cell densities corresponding to about 0.7 mg dry weight/ml. The suspensions were incubated for 15 min at 30 °C with shaking, to allow adaptation to the new medium. Three radioactive amino acids, from the Radiochemical Centre, Amersham, Buckinghamshire, were used: [^{14}C]FPA (5.9 $\mu\text{Ci}/\mu\text{mol}$), [^{14}C]PA (10 $\mu\text{Ci}/\mu\text{mol}$) and [^{14}C]L-leucine (10 $\mu\text{Ci}/\mu\text{mol}$). To each yeast suspension (volume 1 ml) was added labelled PA (0.01 μmol), leucine (0.01 μmol) or FPA (0.1 μmol) as required. After incubation at 30 °C with shaking for the appropriate time, the reaction was stopped by adding an equal volume of ice-cold trichloroacetic acid (TCA; 10 %, v/v).

The amount of incorporation into TCA-insoluble material was measured as follows: To each tube was added 5 ml ice-cold TCA (5 %, v/v) and the suspension filtered through a glass fibre filter (Whatman GFC, 24 mm diam.). The disc was washed five times with 5 ml ice-cold 5 % TCA, then three times with ethanol-ether (1:1, v/v) and finally twice with ether. The filters were dried under an infrared lamp, placed in a phial, immersed in 5 ml of scintillant [butyl-PBD (CIBA-Geigy), 6 g; toluene, 750 ml; methanol, 250 ml] and radioactivity counted with a Packard Tri-Carb liquid scintillation spectrometer. Efficiencies were measured by the internal standard method: known amounts of radioactive amino acids were added to filters prepared under identical conditions to those used for the samples, and were counted in the same way as the experimental samples. A mean value for efficiency (about 65 %) was applied to all the samples of a given experiment.

Assay of oxygen uptake. Oxygen uptake by whole cells was measured polarographically in a Rank-type oxygen electrode coupled to a pen recorder.

Fig. 1. Growth curves of wild-type *S. cerevisiae* strain 10 and a respiratory-deficient (petite) derivative in the presence of FPA at concentrations of 0, 0.1, 0.25, 0.5 and 1.0 mg/ml, in (a) YED medium and (b) YEG medium. ●, Wild type; ○, petite.

RESULTS

Inhibition by FPA

Clearly, the analogue would be most toxic when the growth medium contained no PA. However, since growth of our strains is very limited in a synthetic medium with a non-fermentable carbon source, for the initial tests two series of Petri dishes containing YED and YEG agar with and without FPA were inoculated with 27 strains of *S. cerevisiae*. After 3 days' incubation, growth on each plate was scored. The strains were remarkably similar in their sensitivity to FPA; on YED, most tolerated 0.5 mg/ml with no visible growth but were completely inhibited at the next higher concentration, whereas they were generally more sensitive on YEG and tolerated 0.25 mg/ml.

The results of the Petri dish series were confirmed by measuring growth in liquid media in the presence and absence of FPA. Several strains were tested and Fig. 1 shows typical growth curves obtained for strain 10. Growth in YEG was again inhibited at lower FPA concentrations than growth in YED, indicating that mitochondrial protein synthesis is more sensitive to the analogue than cytoplasmic protein synthesis. This could be explained either by greater incorporation of FPA into mitochondrial protein or by one or more mitochondrial proteins being more affected by incorporation of analogue than cytoplasmic ones. A similar preferential inhibitory effect of the arginine analogue, canavanine, on growth on glycerol has been reported (Wilkie, 1970). From the growth curves (Fig. 1) it was difficult to assess any effect of FPA on respiratory adaptation in the YED shake cultures. Under the standard conditions used (shake culture in a water bath at 30 °C), adaptation generally occurs over a 2 h period from about the 14th to the 16th hour (Fig. 1), and to detect this diauxic phase of the growth curve it is necessary to take a number of extinction readings over this time period. In the presence of FPA, the timing of this stage is difficult since growth is disturbed at the outset (Fig. 1).

Table 1. *Comparison of FPA tolerance of wild-type strains 10, 26 and 27 of S. cerevisiae and their spontaneous resistant mutants on solid fermentable (YED) and non-fermentable (YEG) media*

The mutants were isolated on YED medium. Tolerance indicates the highest analogue concentration permitting growth.

Strain no.	FPA tolerance (mg/ml)	
	YED	YEG
10	0.5	0.25
10-F3	5.0	0.5
10-F5	2.5	0.25
10-F30	5.0	0.25
10-F32	2.5	0.25
10-F34	2.5	0.25
10-F35	2.5	0.25
10-F36	2.5	0.25
10-F39	2.5	0.5
26	0.5	0.25
26-F1	7.5	0.25
26-F2	7.5	0.25
26-F4	7.5	0.25
26-F5	7.5	0.25
26-F7	5.0	0.25
26-F8	7.5	0.25
26-F9	7.5	0.25
27	0.5	0.25
27-F1	5.0	0.5

The inhibitory effect of FPA on the growth of a respiratory deficient (*rho*⁻) mutant of strain 10 is also shown in Fig. 1. Since, in general, there is no mitochondrial protein synthesis in this class of mutant, the effect may be assumed to be entirely due to depression of cytoplasmic protein synthesis. At 0.5 mg FPA/ml, this amounts to about 30 % of the total cellular synthesis. In YEG, on the other hand, growth of *rho*⁺ strains is depressed by about 85 % at this concentration.

Isolation of resistant mutants

Strains 10 and 26 were plated on YED agar containing 1 mg FPA/ml. After 4 to 5 days' incubation, colonies had grown from resistant cells, which were present in the inoculum with a frequency of about 10⁻⁵. Resistant colonies were picked off, purified and retested for resistance on YED and YEG agar containing the full range of FPA concentrations. Only one mutant was isolated from any one culture to ensure that all mutants had arisen independently. During the investigation, the tolerance levels of mutant and parental strains were tested many times and found to be stable, reproducible features. All the mutants showed a marked increase in tolerance to FPA on YED medium, but in many mutants little or no increase in tolerance was found on YEG medium (Table 1). These results were confirmed in liquid media, where a comparison of the growth curves for the mutant 10-F39 with those for the parent strain 10 showed that the mutant tolerated a much higher concentration of FPA in YED medium but only a slightly higher concentration in YEG. This class of mutants was selected for further study, on the assumption that the change to resistance affected only the cytoplasmic protein-synthesizing system and not that of the mitochondrion. None of these FPA-resistant mutants showed increased tolerance to either ethionine (methionine analogue) or canavanine (arginine analogue).

Table 2. Resistance to FPA on solid YED medium in diploids derived from crosses between spontaneous resistant mutants and parental sensitive strains of *S. cerevisiae**

Cross	FPA tolerance (mg/ml)	
	Haploid mutant	Diploid
Resistant $\times$ sensitive*		
10-F3 $\times$ 26	5.0	1.0
10-F5 $\times$ 26	2.5	1.0
10-F32 $\times$ 26	2.5	1.0
10-F35 $\times$ 26	2.5	1.0
10-F36 $\times$ 26	2.5	1.0
10-F39 $\times$ 26	5.0	2.5
26-F1 $\times$ 10	7.5	7.5
26-F2 $\times$ 10	7.5	5.0
26-F4 $\times$ 10	7.5	7.5
26-F31 $\times$ 10	1.0	1.0
26-F40 $\times$ 10	1.0	1.0

* The haploid sensitive parental strains 10 and 26 and the diploid obtained by crossing them have a tolerance of 0.5 mg FPA/ml.

Genetics of resistance

The resistance of diploids derived from crosses between resistant and sensitive strains was assessed on YED agar containing FPA (Table 2). The degree of dominance was greatly influenced by the parent strain from which the resistant mutants were derived: most of those isolated from strain 26 were classified as dominant whereas most from strain 10 were semi-dominant. None of the heterozygous diploids showed complete wild-type sensitivity to FPA. In four mutants examined, each involving a different level of resistance, ascospore tetrads from crosses to sensitive strains segregated 2 resistant:2 sensitive. It was concluded that a single nuclear gene conferred resistance in each case.

FPA tolerance levels of diploids obtained from crosses between pairs of resistant mutants were also determined. The tolerance level of the diploid was always similar to that of the more resistant parent. Tetrad analysis (Table 3) indicated that two resistance genes were segregating in each cross. No evidence for linkage between any of the resistance genes or for linkage of these with the *ade-2* gene, was found.

Incorporation into protein

Since the inhibitory effects of FPA are believed to be caused by the incorporation of the analogue into protein, it might be expected that a resistant mutant would incorporate a smaller proportion of FPA than the wild type under the same conditions, and there are several possible mechanisms by which this could be brought about. Alternatively, if a single protein were particularly susceptible to the conformational distortions brought about by the analogue, a mutational alteration of that protein might make the incorporation of the analogue less deleterious to its function, resulting in resistance. To determine which type of mechanism was operating the incorporation of radioactive FPA and PA into the proteins of several mutants was measured.

As a first step, the effect of FPA on protein synthesis was investigated by measuring leucine incorporation in the presence of the analogue over a 30 min period (Fig. 2). Since the amount of protein synthesized on mitochondrial ribosomes in some of these strains under similar conditions has been calculated as approximately 2% of total cell protein synthesis (Stone & Wilkie, 1974), the mitochondrial contribution to incorporation can be ignored. Protein synthesis was reduced to some extent over this time scale in the

Fig. 2. Inhibition of incorporation of (●) 0.01 mM-[¹⁴C]leucine and (○) 0.01 mM-[¹⁴C]PA into protein of *S. cerevisiae* strain 26 by FPA in YED medium during a 30 min incubation.

Table 3. *Tetrad analysis of crosses between spontaneous mutants of S. cerevisiae resistant to FPA*

All progeny were tested on 2.5 and 5.0 FPA mg/ml in YED medium. In each cross one mutant had a tolerance level of 2.5 mg/ml and the other of ≥ 5.0 mg/ml.

A. Possible tetrad types for different resistance genes.
Maximum FPA concn permitting growth (mg/ml).

Type <i>a</i>	Type <i>b</i>	Type <i>c</i>
5.0	5.0	5.0
5.0	5.0	5.0
2.5	2.5	< 2.5
2.5	< 2.5	< 2.5

B. Observed results

Cross	No. of tetrads		
	Type <i>a</i>	Type <i>b</i>	Type <i>c</i>
26-F7 $\times$ 10-F35	1	3	1
26-F7 $\times$ 10-F5	3	2	1
26-F1 $\times$ 10-F35	1	2	1
26-F1 $\times$ 10-F5	2	2	2

presence of FPA but incorporation of PA was considerably more reduced, showing that FPA specifically replaces PA in protein. Incorporation in these experiments was linear with time for at least 60 min.

Because of the inhibitory effect of FPA on protein synthesis, comparisons were made between parental strains and resistant mutants with respect to analogue incorporation, by expressing this as a fraction of the amount of leucine incorporated during the experiment (60 min). This fraction is proportional to the specific activity of FPA (or PA) in newly synthesized protein. The results, expressed as percentages of parental values, are given in Table 4 for cells grown in YED and YEG. Glucose-grown cells of the resistant mutants incorporated less analogue per unit of [¹⁴C]leucine than the corresponding parental strains. A number also incorporated less externally-added PA than the sensitive parents. These results are consistent with the hypothesis that the cause of growth inhibition is the incorporation of FPA into protein and that resistance is a consequence of the reduction of the amount of that incorporation.

Results obtained with glycerol-grown cells were different, in that some of the mutants differed little from the parental strains. It therefore appears that the resistance mechanism which operates in cells growing in glucose is not always expressed in glycerol. This indicates

Table 4. Incorporation of [^{14}C]FPA and [^{14}C]PA into TCA-insoluble material by mutant and parent strains of *S. cerevisiae* growing in either YED or YEG medium

Incorporation was measured as a fraction of the amount of [^{14}C]leucine (Leu) incorporated by control cultures and is given as a percentage of the value obtained with the parent strain. For strain 26, the absolute measurements for leucine and PA uptake were 1.1×10^4 and 6×10^3 c.p.m./mg protein, respectively. Other parental strains incorporated similarly. Incubation was for 60 min.

Strain	YED medium			YEG medium		
	FPA tolerance (mg/ml)	Incorporation (%)		FPA tolerance (mg/ml)	Incorporation (%)	
		FPA/Leu	PA/Leu		FPA/Leu	PA/Leu
26	0.5	100	100	0.25	100	100
26-F1	7.5	25	38	0.25	104	95
26-F4	7.5	36	52	0.25	57	93
26-F7	5.0	67	83	0.25	100	109
10	0.5	100	100	0.25	100	100
10-F5	2.5	43	93	0.25	70	99
10-F35	2.5	57	82	0.25	109	104
10-F39	2.5	59	87	0.5	76	92

that the assumption that sensitivity in glycerol is a reflection of the selective inhibition of the mitochondrial system is not necessarily always true. The failure to discriminate in glycerol medium in these cases may reflect a resistance mechanism based on selective entry into the cell, selection against the analogue being operative only when glucose is metabolized (see Linstead, Evans & Wilkie, 1974).

Cytochrome synthesis in the presence of FPA

Mutant strain 10-F39 was grown to stationary phase in YED in the presence and absence of FPA (2.5 mg/ml), and cytochrome spectra were obtained in each case. This strain incorporated less analogue than the parent strain 10 but remained sensitive to FPA in YEG medium (Tables 1 and 4). It seemed possible that this sensitivity was due to preferential incorporation of analogue by the mitochondrial system into organellar protein. A relatively high rate of incorporation of analogue by mitochondria would be expected to lead to a depression of organellar protein synthesis (Wilkie, 1970). No significant quantitative differences in the cytochrome spectra of treated and untreated cells could be detected as a possible indication of this. It is possible, however, that although giving quantitatively similar spectra, these proteins could have been functionally deficient following analogue incorporation. Evidence of this was provided by the finding that the rate of O_2 uptake of analogue-grown cells was about 20 % of that of untreated cells. Several polypeptides are known to be synthesized in the mitochondrion and to function in the assembly of the inner membrane complex, e.g. one in particular is believed to effect ATPase attachment (Sierra & Tzagoloff, 1973). Distortion of any of these, after FPA incorporation, could lead to respiratory deficiency.

DISCUSSION

Growth inhibition by FPA correlates with the amount of the analogue incorporated into cellular protein in competition with PA. The initial aim of isolating mutants with a high level of FPA resistance in the cytoplasmic protein-synthesizing system and a sensitive mitochondrial system was achieved, in that these mutants grew in the presence of high analogue concentrations with glucose but not with glycerol as carbon source. Some

of the mutants incorporated significantly less FPA into cell proteins compared with parent strains, whether glucose or glycerol was being metabolized. The tentative conclusion may be drawn that in these strains the mutation enabled the cytoplasmic system to discriminate against FPA without any change occurring in the sensitivity of the mitochondrial system. It remains to be established whether this sensitivity is due to a relatively greater substitution of PA by FPA into mitochondrial polypeptides generally (which would reflect a higher affinity of the organellar tRNA-activating enzyme complex for FPA, compared with the cytoplasmic complex), or whether a particular mitochondrial polypeptide(s) is more crucially affected by conformational distortions resulting from FPA incorporation.

In a second category of resistant mutant, the amount of FPA incorporated into cellular proteins varied with the carbon source, less analogue being incorporated in a glucose medium than in a glycerol one. Possibly a mutation affecting cell permeation to FPA had occurred, but was operative only when glucose was being metabolized.

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