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Variations in enzyme phenotypes and their underlying genotypes among wild strains of the genus *Neurospora*

By M. GRINDLE* and R. H. DAVIS

Department of Botany, University of Michigan, Ann Arbor, Michigan, 48104, *U.S.A.*

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SUMMARY

Twenty-three exotic strains of four species of *Neurospora* (*N. crassa*, *N. sitophila*, *N. intermedia* and *N. tetrasperma*) were grown in minimal medium and their enzyme phenotypes were compared. The exotic strains did not differ substantially with respect to the specific activities of the biosynthetic enzymes, ornithine transcarbamylase (OTC), carbamyl phosphokinase and aspartate transcarbamylase. Exotic strains of *N. crassa* and *N. sitophila* were crossed with laboratory mutants of *N. crassa* to determine whether there were significant differences among the exotic strains in the gene complexes underlying their enzyme phenotypes. Most of the exotic strains carried similar OTC structural genes and similar genetic modifiers of OTC activity: their OTC structural genes were expressed normally in the genomes of the parent strains, in the alien genomes of standard laboratory mutants and in the mixed genomes of parent × mutant hybrids. One exotic *N. crassa* carried a distinctive OTC structural gene that elevated OTC activity when transferred into the genome of a standard laboratory mutant. A second exotic *N. crassa* carried distinctive genetic modifiers of OTC activity: two mutations, c^- and *hi*, interacted in the exotic parent to normalize OTC activity and rate of growth, but *hi* led to extremely high OTC activities and to a reduction in rate of growth when separated from c^- by interstrain recombination.

1. INTRODUCTION

Isolated populations of the same species gradually become differentiated due to independent mutations. One might expect some mutations to be suppressed by compensating non-allelic changes and, as a result, numerous self-effacing epistatic interactions might arise in the process of evolutionary divergence. Mutations of the structural gene for a particular enzyme, for example, might be compensated by mutations at other gene loci affecting the expression of the structural gene. Should different combinations of structural and regulatory gene mutations prevail among isolated populations, the regulatory gene products of one population might be incompatible with the structural genes of another, even though the phenotypes are

* Present address: Department of Genetics, The University, Sheffield S10 2TN, Yorkshire, England.

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similar. The extent to which phenotypically similar wild strains can differ genetically, however, is not clear (see Dobzhansky, 1951; Grant, 1964; Mayr, 1963; Stebbins, 1966; Waddington, 1957, for reviews).

Questions relating to genetic and phenotypic divergence can be approached experimentally with species of *Neurospora*. The organisms are haploid, interspecific crosses may be fertile, and genetic knowledge of one species, *N. crassa*, is extensive. Metabolic and enzymic analysis of wild strains permit evolutionary study at the most fundamental level of the phenotype (see Bodmer & Parsons, 1962). We chose the specific activities of three biosynthetic enzymes as the characters to be surveyed throughout the genus and to be analysed more fully in terms of genetic and phenotypic divergence between the species *N. crassa* and *N. sitophila*. Our major intent was to determine whether a structural gene of one species would be expressed normally in another species' genome. We hoped also to reveal any serious metabolic disharmonies indicative of genetic differences between and within species. An abstract of some of this work has appeared previously (Grindle & Davis, 1966).

2. EXPERIMENTAL APPROACH

The enzymes studied were the arginine enzymes, ornithine transcarbamylase (OTC) and carbamyl phosphokinase (CPK, a component of the arginine-specific carbamyl phosphate synthetase system), and the pyrimidine enzyme, aspartate transcarbamylase (ATC). The structural genes responsible for the three enzymes are *arg-12* (OTC), *arg-3* (CPK) and *pyr-3* (ATC). Previous genetic and physiological work on the mutants has been reviewed by Davis (1967). The parameter used in our studies is the specific activity of the enzyme in question in crude, dialysed extracts; we assume that this character is underlain by a number of genes, of which the structural gene is one.

The first phase of our analysis is descriptive: the OTC, CPK and ATC activities of wild strains of four species of *Neurospora* (*N. crassa*, *N. sitophila*, *N. intermedia* and *N. tetrasperma*) were compared.

In the second phase of our analysis wild strains were crossed with laboratory mutants of *N. crassa* to reveal whether there were significant differences among the wild strains in the gene complexes underlying their enzyme phenotypes. Consider, for example, a mutant unable to synthesize OTC: it carries an inactive allele of the OTC structural gene (*arg-12*$^-$) and a number of hypothetical genes (x) that can modify the expression of the structural gene. An exotic wild strain carries an active allele of the structural gene (*arg-12*$^+$) and, perhaps, a different set of modifiers (y) allelic with the set of x modifiers of the laboratory mutant. The mutant (*arg-12*$^-$, $x_1 \ldots x_n$) is crossed with a wild strain (*arg-12*$^+$, $y_1 \ldots y_n$) and prototrophic progeny are sampled for enzyme analysis. These prototrophs carry the *arg-12*$^+$ allele of the structural gene but they have a mixed set of modifiers, averaging half x and half y. The f_1 generation (roughly equivalent to the F_2 of diploids) will therefore display interactions among x and y modifiers affecting the expression of the *arg-12*$^+$ allele. Extreme variants can be analysed further. On backcrossing f_1 prototrophs to the

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arg-12$^-$ mutant and selecting only prototrophic progeny, the *arg-12*$^+$ allele from the wild parent is retained but the genetic modifiers, *y*, of the wild parent are gradually replaced by *x* modifiers of the laboratory mutant. The final backcross progeny, if phenotypically homogeneous, will indicate whether the *arg-12*$^+$ allele from the wild parent is expressed normally in the genetic background of the laboratory mutant. Pedigrees were developed in this way to analyse and compare the OTC, CPK and ATC structural genes of several wild strains.

The data from such a crossing programme must be qualified by three major factors. First, homogeneity of f_1 progeny may indicate selection against variants rather than their absence. Secondly, modifiers that are strongly linked to the structural genes will be retained during backcrossing. Thirdly, minor variants may be overlooked because our assay methods discriminate only those isolates with an enzyme activity about 50 % greater or less than normal.

3. MATERIALS AND METHODS

(*a*) *Strains.* The wild strains listed in Table 1 were obtained from the Fungal Genetics Stock Centre, Dartmouth College, New Hampshire, U.S.A. (strains 349–965), from Dr J. R. S. Fincham (strains 2 and 3) and from Dr A. M. Srb (strain 10).

The laboratory mutants used in crosses with the exotic wild strains were as follows. (i) *arg-12*; alleles *arg-12*^{s} and UM-3—mutants partially and completely deficient in OTC activity, respectively (Davis, 1962*a*, *b*; Davis & Thwaites, 1963). The *arg-12*^{s} mutation, which imposes no growth requirement despite a 95–98 % reduction in OTC activity, can be identified by a rapid semi-quantitative test for OTC. The UM-3 allele imposes an absolute requirement for arginine. (ii) *arg-3*; allele 30300—an arginine-requiring mutant completely deficient in CPK activity (Davis, 1963, 1965*a*, *b*). (iii) *pyr-3*; alleles KS-36 and KS-43—uridine-requiring mutants lacking ATC activity (Davis, 1960). The mutants are closely related by origin or backcrossing to the *N. crassa* laboratory wild type, 74*A*.

All stocks were maintained on agar slants of minimal medium (Vogel, 1964) supplemented, where appropriate, with 100 μg uridine or 200 μg L-arginine HCl per ml.

(*b*) *Crosses.* Strains were crossed on the synthetic medium of Westergaard & Mitchell (1947) and on Difco cornmeal agar, at 25 °C. At least 2 weeks after fertilization, ascospores were heat-shocked at 60 °C and plated on Vogel's minimal medium. After 6–8 h at 30 °C, germinated ascospores were transferred to tubes of Vogel's minimal medium and a random sample of the prototrophic isolates was analysed.

Crosses involving *N. crassa* 683 were also analysed in more detail by plating ascospores on arginine-supplemented Vogel's medium containing 1 % sorbose, 0·05 % fructose and 0·05 % glucose as carbon sources. After 18 h at 30 °C, germinated spores were transferred to tubes of arginine-supplemented Vogel's medium.

(*c*) *Growth, extraction and enzyme assay.* The exotic strains and interstrain progeny were grown and harvested as outlined by Davis & Mora (1968). During

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logarithmic growth in aerated liquid medium, samples of mycelia were removed at different times and acetone-dried on filter paper. Each sample was weighed, extracted in 2–5 ml cold 0·02 M–K^+ phosphate buffer and assayed for enzyme activity as described by Davis (1965*c*). Activities are expressed as μmoles product per mg protein per hour at 25 °C (OTC and ATC) or 35 °C (CPK).

Washed, moist mycelia, extracted in 5 % perchloric acid, were used for arginine determinations by the method of Van Pilsum, Martin, Kito & Hess (1956).

4. RESULTS

(*a*) *Sexual fertility*. The arginine and pyrimidine mutants were mated to 14 strains of *N. crassa* and to 8 strains of *N. sitophila*. Crosses of *N. crassa* 683 with arginine mutants were very poor but most *N. crassa* × *N. crassa* crosses produced numerous viable ascospores after 2–3 weeks. The *N. crassa* × *N. sitophila* crosses were either sterile or they produced a few viable ascospores after 6–8 weeks. Crosses with *arg-12*^{s} were often more productive than those with UM-3.

(*b*) *Enzyme variations among replicates of the laboratory wild-type 74A*. To assess whether the variations in enzyme activity among exotic strains were statistically significant we required an estimate of the experimental errors incurred during analysis of our material. We also needed to know whether the enzyme activities of the exotic, f_1 and backcrossed strains differed significantly from that of the wild-type strain (74*A*) whose genetic background was closely related to the mutants used in the crossing programme. Strain 74*A*, therefore, was grown, harvested and assayed for enzyme activity on three separate occasions. A total of 14 mycelial samples (which we shall refer to as replicates), that were obtained during logarithmic growth, were analysed. The mean specific activities of OTC, CPK and ATC and their variances of the 74*A* replicates are given in Table 1.

(*c*) *Enzyme variations among exotic strains*. The OTC, CPK and ATC activities of the exotic strains are shown in Table 1. Each of the figures given in the Table is the mean specific enzyme activity of at least two replicates. Most of the enzyme activities fall within a narrow range and the exceptions are not restricted to any one species. With respect to all three enzymes, the mean specific activity of the total generic sample is almost identical to the mean of the 74*A* replicates, and the variances of the generic and replicate samples are not significantly different at the 5 % level.

(*d*) *Enzyme phenotypes of* f_1 *and backcross progenies*. Each of the *N. crassa* and *N. sitophila* wild strains was crossed with UM-3, *arg-12*^{s}, *arg-3* and *pyr-3* mutants, and small samples of f_1 progeny were tested for enzyme activity. None of the progeny had grossly abnormal OTC, CPK or ATC activities. We therefore concentrated on OTC activity in crosses of wild strains with UM-3 and *arg-12*^{s}.

The mean OTC activities, standard deviations and sample sizes of f_1 and backcross progenies are given in Table 2. Most of the progenies are not significantly different in variance or mean from those of the 74*A* replicates. There were two notable exceptions: backcross progeny from the 430 × *arg-12*^{s} pedigree had a

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Table 1. *Origin, and specific activities of the enzymes, ornithine transcarbamylase (OTC), carbamyl phosphokinase (CPK) and aspartate transcarbamylase (ATC) of wild strains of* Neurospora

Species	Strain no.* and mating type	Origin†	Specific activity‡ OTC	CPK	ATC
N. crassa	429 a	Puerto Rico	9·4	0·20	2·7
	430 A	N. Africa	11·2	0·13	4·2
	431 A	Java	13·2	0·18	3·2
	433 a	Phillipines	6·0	0·14	3·9
	434 A	Liberia	13·3	0·16	3·7
	436 a	Singapore	11·1	0·15	3·7
	683 a	Louisiana	16·0	0·15	4·5
	687 a	(Abbott)	10·5	0·19	—
	757 A	Louisiana	17·3	0·28	—
	851 A	Costa Rica	11·5	0·13	2·3
N. sitophila	2 a	England	9·3	0·22	2·3
	3 A	England	10·0	0·17	2·8
	10 A	N. Africa	7·6	0·08	1·6
	415 A	(Dodge)	9·2	0·14	2·7
	417 A	(Dodge)	11·7	0·20	3·2
	573 a	?	11·1	0·14	2·1
	848 a	(McCrea)	8·7	0·32	—
N. intermedia	629 a	Phillipines	11·2	0·17	2·2
	962 a	(Tai)	12·3	0·16	3·0
N. tetrasperma	349	(Sussman)	11·4	0·11	—
	590	(Dodge)	11·9	0·02	4·0
	850	Honduras	10·6	0·14	3·6
	965	Liberia	11·7	0·10	5·7

* Except for strains 2, 3 and 10, strains are designated by their Fungal Genetics Stock Centre (FGSC) numbers.

† Laboratory sources given in parentheses; remainder show geographic origin.

‡ Means ($\bar{x}$) and variances (V) of specific activities for total generic sample are as follows ($\bar{x}$ and V for 14 replicates of the standard laboratory wild type, 74*A*, given in parentheses): OTC—$\bar{x}$ = 11·1 (11·3), V = 5·92 (3·6); CPK—$\bar{x}$ = 0·160 (0·167), V = 0·0038 (0·0014); ATC—$\bar{x}$ = 3·2 (3·4), V = 0·99 (0·55).

significantly different mean OTC activity and f_1 progeny from the 683 × UM-3 pedigree had a significantly different variance.

The mean OTC activity in the 430 × *arg-12*^{s} pedigree increased gradually on backcrossing and reached 17·8 in the b_4 progeny compared with 12·9 of the f_1 progeny and 11·2 of the 430 parent strain (P for difference between mean OTC activities of f_1 and b_4 < 0·001). The variances of the f_1 and b_4 progenies were not significantly different. The abnormally high OTC activities of b_4 isolates were substantiated by contemporaneous replicate assays of b_4, 430 and 74*A* cultures. Since the OTC structural genes of all the exotic strains, except 430, were expressed normally when backcrossed into *arg-12* mutants (Table 2), 430 appears to be the only strain with a distinctive *arg-12*$^+$ allele. The effects of the distinctive *arg-12*$^+$ allele may have been masked in the 430 parent by mutations affecting protein

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extractability. The protein extractability of b_4 isolates, which was normal, was 70 % greater than that of 430.

Strain 683 lacked conidia and was used as the protoperithecial parent in a cross with UM-3. Few ascospores were obtained from the cross but they germinated well on arginine-supplemented medium. Out of 100 germinated spores tested, 14 did not grow further on either minimal or supplemented medium and 31 required arginine for growth. The 55 prototrophic progeny could be partitioned into two non-overlapping groups, 40 having 'normal' OTC activities (9·4–22·8; mean = 14·9)

Table 2. *Specific activities of the enzyme ornithine transcarbamylase (OTC) of wild strains of* Neurospora crassa *and* N. sitophila *and of first-generation and backcross progenies from crosses of the wild strains with the arginine mutant UM-3 or* arg-12^s

Wild strain and OTC activity		OTC specific activity								
		f_1 progeny			b_2 progeny			b_4 progeny		
		$\bar{x}$	σ	N	$\bar{x}$	σ	N	$\bar{x}$	σ	N
N. crassa										
429*	*9·4*	*11·0*	3·4	7	*11·4*	1·6	8	—	—	—
430†	*11·2*	*12·9*	2·1	12	*14·7*	5·3	6	*17·8*	3·3	23
436*	*11·2*	*12·2*	4·5	7	*11·2*	1·8	6	—	—	—
683*	*16·0*	*24·4*‡	18·2	55	*13·3*	3·7	18	—	—	—
851†	*11·5*	*10·1*	2·0	6	*11·2*	1·0	6	—	—	—
N. sitophila										
3†	*10·0*	*11·8*	4·8	10	*11·4*	0·3	6	—	—	—
415†	*9·2*	*9·9*	1·5	8	*10·9*	3·3	6	—	—	—
417†	*11·7*	*8·7*	1·7	7	*11·1*	1·0	6	—	—	—

* Wild strains were crossed with UM-3. † Wild strains were crossed with *arg-12^s*.
‡ Bimodal distribution (see text).
$\bar{x}$ = Mean specific activity, σ = standard deviation, N = number of progeny tested ($\bar{x}$ and σ for replicates of the standard wild type, 74*A*, = *11·3* and 1·9, and for the total generic sample in Table 1 = *11·1* and 2·45).

and 15 having 'high' OTC activities (31·3–94·8; mean = 49·6). The isolates with 'normal' OTC activities included both conidial and aconidial strains, all of which had normal rates of growth. The isolates with 'high' OTC activities were all conidial and they grew poorly in liquid media.

Genetic and physiological analysis of f_1 progeny showed that OTC activity was influenced by many genes, and three genes with major effects on OTC activity were identified. The data are compatible with the following interpretation:

(i) Strain 683 carries an active allele of the OTC structural gene, a mutation c^-, for 'conidia-less', and a mutation, *hi*, for 'high-OTC' activity (genotype = *arg-12*$^+$, c^-, *hi*). Strain UM-3 carries an inactive allele of the OTC structural gene and the normal alleles of the 'conidia-less' and 'high-OTC' genes (genotype = *arg-12*$^-$, c^+, *hi*$^+$). The three genes are unlinked.

(ii) The *hi* mutation elevates OTC activity and causes a 30–40 % reduction in rate of growth when separated from the c^- mutation (genotype of progeny with

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'high' OTC = *arg-12*$^+$, *c*$^+$, *hi*). Additional genes responsible for the variations in OTC activity among the 15 'high' OTC f_1 isolates have not been identified.

(iii) The *c*$^-$ mutation interacts with *hi* to normalize OTC activity and rate of growth (genotypes of progeny with 'normal' OTC activities = *arg-12*$^+$, *c*$^-$, *hi* and *arg-12*$^+$, *c*$^-$, *hi*$^+$ and *arg-12*$^+$, *c*$^+$, *hi*$^+$). The numbers of f_1 progeny with 'normal' OTC activities differed significantly from the expected $2c^-:1c^+$, however, and additional genetic modifiers of OTC activity are not excluded.

(iv) The *hi* mutation, when combined with the *arg-12*$^-$ mutation in further crosses, resulted in a phenotype unable to grow on supplemented medium. This accounts for the 14 germinated spores of the 683 × UM-3 cross that failed to grow when transferred to fresh media (presumed genotypes = *arg-12*$^-$, *c*$^-$, *hi* and *arg-12*$^-$, *c*$^+$, *hi*). The implied assimilatory defect due to *hi* was confirmed by arginine-uptake studies of *arg-12*$^+$, *hi* strains and is consistent with the observation that *arg-12*$^+$, *hi* strains were resistant to the amino acid analogue, *p*-fluorophenylalanine.

(v) The *hi* mutation causes an increase in CPK activity (of the arginine pathway) but does not affect ATC activity (of the pyrimidine pathway).

Phenotypically normal f_1 and b_1 isolates (genotype = *arg-12*$^+$, *c*$^+$, *hi*$^+$) were used as the parents in further crosses with UM-3. The final backcross progeny had normal OTC activities (Table 2), demonstrating that the *arg-12*$^+$ allele of 683 is expressed normally when transferred into the genetic background of the laboratory mutant.

5. DISCUSSION

This work demonstrates that wild strains of *Neurospora* grown in minimal medium have similar OTC, CPK and ATC activities, and that many wild strains of *N. crassa* and *N. sitophila* carry similar OTC structural genes and similar genetic modifiers of OTC activity. The enzymes studied here have been shown to display clear regulatory responses to starvation, so the constancy of enzyme phenotypes of exotic strains and of progeny derived from them by crossing to laboratory mutants cannot be attributed to an inherent invariance of structural gene expression. These data suggest that there has been selection against extreme variants during evolutionary divergence of the genus, *Neurospora*, resulting in stabilization of the enzyme genotypes, as well as the enzyme phenotypes, of isolated populations. However, relatively small numbers of interstrain hybrids were analysed and significant enzyme variants may have been overlooked. We also cannot rule out strong linkage of genetic modifiers to the structural genes and selection against modifiers during our crossing programme.

The recovery of progeny with exceptional enzyme activities from two interstrain crosses indicates that a few, significant genetic differences persist among phenotypically similar wild strains. The release of variation by interstrain recombination also validates our method for revealing regulatory variants. One exotic strain of *N. crassa* (430) carried a distinctive OTC structural gene: its *arg-12* gene was expressed normally in the parental genome but led to abnormally high OTC activities when transferred into the genome of a standard laboratory mutant. A second exotic

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strain of *N. crassa* (683) carried distinctive genetic modifiers of OTC activity: its *arg-12* gene was expressed normally in the genomes of the parental and laboratory mutant strains but not in the genomes of certain parent × mutant hybrids. Two genetic modifiers, c^- and *hi*, interacted in the 683 parent to normalize OTC activity but the *hi* mutation led to extremely high OTC activities when separated from c^- by interstrain recombination. Thus, a mutation in the wild strain has evidently been compensated by a second mutation at a different locus. It may be significant from an evolutionary standpoint that the c^-, *hi* parent with normal OTC activity had a considerable growth-rate advantage over c^+, *hi* hybrids with extremely high OTC activities. The physiological mechanisms underlying the exceptional strains are not known but, in view of more systemic effects associated with them, they are probably not involved specifically with the regulation of OTC.

Our data are consistent with those from studies on the interchangeability of genome components among related forms of bacteria (e.g. Falkow, Wohlhieter, Citarella & Baron, 1964; Signer, 1965; Sarker, 1966; Somerville, 1966; Jacoby & Gorini, 1967). There have been few similar investigations of eucaryotes (e.g. genetics of tumour formation in *Nicotiana* (Kehr, 1965; Smith, 1968)) and, in particular, very little is known of the regulation of individual enzymes. The rationale outlined in this report may be one of the few means of obtaining variants of specific regulatory systems in eucaryotes. In addition, further work of this sort should reveal whether there are significant differences in variation between anabolic and catabolic enzymes, between the various components of the regulatory system and between haploid and diploid organisms.

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