STRUCTURAL GENE FOR ORNITHINE TRANSCARBAMYLASE IN NEUROSPORA

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Received July 17, 1963

THE conversion of ornithine to arginine in Neurospora is similar to that demonstrated in many other organisms. This route involves the enzyme ornithine transcarbamylase (OTC), which catalyzes the combination of carbamyl phosphate (CAP) and ornithine to form citrulline, with the liberation of inorganic phosphate. Citrulline is subsequently converted to arginine in two successive enzymatic steps, the intermediate being argininosuccinic acid. Until the arginine enzymes were studied in Neurospora mutants, the nutritional phenotypes of these mutants seemed straightforward (SRB and HOROWITZ 1944). There were mutants which responded to ornithine, and to citrulline and to arginine ("*orn*" mutants), others which responded to citrulline and to arginine ("*cit*" mutants) and others which responded to arginine only. A number of difficulties were encountered, however, in subsequent studies of *orn* mutants, as none appeared to lack enzymes whose absence might be expected on the basis of their nutritional phenotypes (FINCHAM 1960; VOGEL 1955; VOGEL and KOPAC 1959). Other hypotheses regarding the action of these mutant genes were devised in terms of coordination of enzyme sequences *in vivo* rather than in terms of simple genetic blocks (VOGEL 1955; VOGEL and KOPAC 1959; DAVIS 1962a). Similar difficulties were encountered in an analysis of mutants at the *cit-1* and *cit-2* loci, all of which could be shown to have the very enzyme that at least some would be expected to lack, namely, OTC (FINCHAM 1960; REISSIG 1960; DAVIS 1962a). Together with this observation was the fact that no OTC-less mutants had been found among large numbers of arginine-requiring mutants. A new search for this type of mutant is described here; similar work is being done in the laboratory of WOODWARD (1963). An abstract of this work has appeared previously (DAVIS and THWAITES 1963).

A priori, a citrulline requirement which is not satisfied by ornithine would be expected to arise in one of several ways. One is a limitation in CAP synthesis, a possibility that must take into account the role of CAP in pyrimidine synthesis. This possibility seems to bear on mutants of both *cit* loci, and has been discussed extensively (CHARLES 1962; DAVIS 1960, 1962a; REISSIG 1960; REISSIG 1963). Moreover, *cit-1* has been shown in this laboratory to lack completely a carbamate kinase demonstrable in extracts of wild type. (The fact that *cit-1* does not require pyrimidines is circumstantial evidence for the existence of a pyrimidine-specific source of CAP, as yet undetected [DAVIS, unpublished]). A citrulline requirement could also arise through a lack of OTC. The only mutation known to affect

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OTC is a gene, *s*, which is recognizable by its suppressor interaction with certain *pyr-3* mutants (DAVIS 1961). This mutation leads to a 97 percent reduction in OTC activity and an alteration of its kinetic properties (DAVIS 1962a, 1962b); the limitation of arginine synthesis resulting from *s* is not sufficient to limit growth rates significantly in the absence of arginine (DAVIS 1962a). In view of the above discussion, quite a number of questions may be asked. The present experiments were designed to determine (a) whether OTC was dispensable in regard to citrulline formation; (b) whether two forms of OTC were present in wild-type Neurospora (DAVIS 1962b), specified by different loci (the *s* locus being one, and, conceivably, *cit-1* or *cit-2* being another) or lastly, (c) whether the *s* mutation was the only known mutant allele of a single structural locus for OTC. The tests involved crosses of *s* with mutants representing the *cit-1* and *cit-2* loci, isolation of OTC-less mutants from *s*, and isolation of OTC-less mutants from wild-type.

MATERIALS AND METHODS

The strains of Neurospora used were *cit-1* (= *arg-3:* 30300), *cit-2* (= *arg-2:* 33442), *orn-2* (= *arg-6:* 29997), *orn-3* (= *arg-7:* 34105), wild types 73a, 74A, 5297a, and 25a, and strains carrying the *s* mutation (7d and 15b; cf. DAVIS 1962a). Other strains, derived from *s* and wild-type 74A by mutation, are described in the next section.

Crosses were performed on the synthetic crossing medium of WESTERGAARD and MITCHELL (1947). All other media utilized the basic minimal medium of VOGEL (1956), with 1.5 percent sucrose unless otherwise indicated. Colonial growth in agar media was obtained by substituting 1 percent sorbose and 0.2 percent sucrose for sucrose where conidia were plated; for nutritional analysis of ascospore progeny, 0.8 percent sorbose plus 0.4 percent sucrose were used in spot testing.

Mutants were isolated after dry conidia had been treated for 0.5 to 1.5 minutes with ultraviolet irradiation by an 8W germicidal lamp at 15 cm. After exposure, the conidia were suspended in 100 ml unsupplemented medium, contained in a 250 ml flask, at concentrations of 10^5–10^6 conidia per ml. The flask was shaken on a reciprocating shaker (90 cycles per minute, 1.5 inch stroke) for approximately 60 hours with filtrations through double-layered cheesecloth at 17, 24, 30, 36, 42, 48, 54, and 60 hours after treatment (WOODWARD, DE ZEEUW and SRB 1954). The conidia which remained were concentrated by centrifugation and plated on sorbose medium supplemented with 100 μg arginine HCl per ml. Colonies were isolated in tubes of arginine-supplemented medium and were tested subsequently on minimal medium to identify arginine-requiring strains. Nutritional tests were carried out on the arginine intermediates ornithine and citrulline in small volumes of liquid medium. They were scored after 5 days incubation at 28°.

Complementation between pairs of mutants was carried out in 1 to 2 ml minimal medium by inoculation with drops of the appropriate conidial suspensions. They were scored after 2 and 4 days; the data are interpreted in terms of the num-

ber of mutant loci represented in the spectrum of mutants isolated. No attempt was made to observe intragenic complementation.

Enzymatic analysis for OTC was done in two general ways depending upon the type of data desired. (1) For specific activity measurements of OTC, strains were grown and harvested according to the method of DAVIS and HAROLD (1962). Assays were carried out on dialyzed acetone powder extracts as described previously (DAVIS 1962a). Specific activities are reported as micromoles citrulline per mg protein per hour. The normal activity of wild-type mycelia was approximately 15; of strains carrying the *s* mutation, 0.5 to 1.0 (DAVIS 1962a). (2) For determining ratios of enzymatic phenotypes in large numbers of isolates, qualitative tests were used. Each isolate was grown for 2 to 3 days in 10 ml arginine-supplemented medium with 2 percent sucrose. The mycelia were harvested and dried quickly with acetone on a small Buchner funnel, transferred to a centrifuge tube and extracted with 2 ml 0.02 M Tris-acetate buffer, pH 8.6. Extracts (0.2 ml) were tested in reaction mixtures as described by DAVIS (1962b) scaled down to one half or one quarter the normal volume.

To distinguish wild-type ("normal") from *s* ("low" OTC) or OTC-less strains, reactions of one-fourth normal volume were allowed to proceed for 10 minutes. The mixtures were not deproteinized; the reactions were stopped by the addition of 0.5 ml of the phosphoric-sulfuric acid-diacetylmonoxime color reagent described by ARCHIBALD (1944) for citrulline. The tubes were heated at 60°C for one hour, and classified as deeply colored, diagnostic of wild-type OTC activity, or without color, diagnostic of low or no OTC activity in the original extracts. To distinguish the low OTC activity characteristic of *s* from the complete absence of OTC, incubation mixtures for the OTC reaction were scaled down to one-half normal volume; reactions proceeded for one hour, and were stopped by the addition of 0.25 ml 2 M perchloric acid. One ml of each of the supernatants was then tested in the color reaction described by KORITZ and COHEN (1954), as modified by DAVIS (1962b), scaled down by one half. In this test, reaction mixtures using *s* extracts gave a deep color; those using OTC-less extracts gave virtually none.

RESULTS

Crosses of orn *and* cit *mutants with* s: When *s* was crossed with *cit-1* or *cit-2*, four classes of progeny were regularly found. The two prototrophic classes were wild type and *s*, and were differentiated by qualitative analysis of OTC. Two arginine-requiring classes ($arg^- s^+$ and $arg^- s$) could be distinguished in the same way. Upon further analysis, the specific activities of the OTC of *cit-1 s* and *cit-2 s* were found to be characteristic of, or somewhat higher than, the *s* siblings (Table 1). This is also true of *orn-1 s*, as reported previously (DAVIS 1962a), and of *orn-2 s* and *orn-3 s* in further experiments (Table 1). This being the case, the hypothesis that one of these arginine mutants is an OTC mutation, which when combined with *s* leads to the complete absence of this enzyme, is shown to be invalid. There is no evidence of linkage between *s* and *orn-2*, *orn-3*, *cit-1*, or *cit-2*.

TABLE 1

*OTC activities of the progeny of various crosses**

Cross	*arg*+ *s*+	*arg*+ *s*	*arg*− *s*+	*arg*− *s*
s × *cit-1*	normal	0.53	normal	0.52
s × *cit-2*	normal	0.62	normal	1.09
s × *orn-2*	normal	0.53	normal	0.53
s × *orn-3*	normal	0.50	normal	1.14

* Normal OTC = greater than 10 micromoles citrulline per mg protein per hour as determined by qualitative OTC assay; the specific activities of isolates carrying *s* are average activities of three or four isolates from the crosses listed.

OTC-less mutants derived from s: Using conidia of the *s* strain (7d) for starting material, arginine-requiring mutants were isolated after ultraviolet treatment. Those able to grow on citrulline but not on ornithine were saved for analysis. Sixty-five such mutants were isolated; they fell into six to eight complementation groups, when tested in pairs. (Two groups are represented by weak strains whose complementation responses are ambiguous.) One or more members of most complementation groups were grown in a quantity sufficient for OTC analysis; representative specific activities, given in Table 2, show that mutants of one group lack measurable OTC activity. It is probable that mutants in the other complementation groups include the double mutants *orn-1 s*, *orn-2 s*, *orn-3 s*, *arg-4 s*, *cit-1 s* and *cit-2 s;* it has been shown (MITCHELL and MITCHELL 1952; and confirmed by us) that the *orn-1*, *orn-2* and *orn-3* mutants will not utilize ornithine for growth if the *s* mutation is also present (cf. DAVIS 1962a). The *arg-4* mutant (21502) is also an ornithine-requiring mutant, but nutritional data on the *arg-4 s* double mutant are not available. The genetic constitution of mutants containing OTC was not studied further; WOODWARD (unpublished) has performed a genetic analysis of a similar series of mutants derived from *s*.

TABLE 2

OTC activities of citrulline-requiring mutants isolated from s

Mutants	Number isolated	Representative OTC activity	Mutants tested for OTC
Complementation Group I	9	1.02	3
II	15	<0.02	14
III	3	0.89	1
IV	22	1.12	2
V	8	1.35	5
VI	2	1.54	1
VII*	1	. . .	. .
VIII*	2	. . .	. .
Original *s* strain (7d)		0.50	. .
Wild type 5297a		15.3	. .

* These groups are not well distinguished from the others; their growth was very spare. There was some difficulty in distinguishing whether complementation or partial growth on minimal medium was responsible for their complementation response.

The isolation of OTC-less mutants with the expected nutritional requirement strongly supports the indispensability of OTC in arginine metabolism in Neurospora. The genetic basis of this phenotype was then investigated.

The OTC-less mutant UM-3, when mated to an *s* strain of the opposite mating type (15b) gave a progeny ratio of 91 prototrophs to 95 arginine-requiring. Qualitative enzyme assays of both classes showed the former to contain OTC activity characteristic of the *s* parent and the latter to contain no OTC at all. It is concluded that the OTC-less mutant, as expected, has a single-gene difference from the *s* strain from which it is derived.

The OTC-less mutant UM-3, when mated with wild type 25a (normal OTC), gave a progeny ratio of 94 prototrophs to 98 arginine-dependent. Of the former class, all had wild-type levels of OTC; the latter had none. The *s* phenotype (prototrophic with low OTC) was not recovered in these crosses. A similar cross of UM-3 and wild type 5297a gave a ratio of 108 prototrophs to 83 arginine-dependent (P for 1:1 segregation = 0.05–0.1). Of the former class, all had wild-type OTC levels; that is, the original *s* phenotype was not recovered.

From these data, it is concluded that the OTC-less phenotype, when derived from the *s* strain, is probably referable to a further mutation at the *s* locus, since the original *s* phenotype could not be recovered in crosses of UM-3 to wild type. The other 14 OTC-less mutants isolated from *s*, on the basis of their enzymatic and complementation phenotypes, are assumed to be allelic to UM-3 and thus to *s*, although genetic evidence available proves only that they are single-factor mutants. A test for reversion of any of these OTC-less mutants to prototrophy was negative.

Enzymatic analysis shows that OTC activities of wild type, *s* and UM-3 are additive; that is, there are no inhibitory or stimulatory interactions in mixed extracts of these three strains.

OTC-less mutants derived from wild type: From the genetic analysis of OTC-less mutants isolated from *s*, a single locus for OTC was inferred. It was then curious that, despite repeated efforts of others since 1941, no OTC-less mutants had been derived from wild type. A search for OTC-less mutants among irradiated conidia of wild type 74A was performed. Twenty-five citrulline-requiring mutants were isolated, and a sample indicated that three complementation groups were present. Groups I and II were approximately equal in number, and these mutants could grow slowly on minimal medium. The third complementation group (Group III) was defined by a single absolute mutant, UM-107. When the mutants were tested for OTC activity, the mutants of Groups I and II were found to contain normal or very high levels of this enzyme. These probably represent the *cit-1* and *cit-2* loci. (Mutants of the ornithine-requiring category, because they could grow on ornithine, were not represented in this spectrum of mutants. This is in contrast to *orn* mutants isolated in a genetic background containing *s*.) The third complementation group, represented only by UM-107, lacked this enzyme completely.

The possibility that UM-107 was a contaminant derived from OTC-less strains previously isolated from *s* was checked by testing the ability of such a strain

(UM-3) to form a complementing heterokaryon with UM-102 and UM-103, representing groups I and II of the mutants selected from 74A. UM-3, in contrast to UM-107, failed to complement with UM-102 and UM-103, presumably because of the heterokaryon incompatibility differences between the genetic background of *s* and that of the wild type from which UM-102, UM-103 and UM-107 were isolated. It was thereby concluded that UM-107 had been derived from wild type 74A. The success in isolating an OTC-less mutant from wild type, in view of their absence in other selection experiments (FINCHAM 1960), must be looked upon as freakish.

A mating of UM-107 with wild type 5297a yielded 96 prototrophic to 101 arginine-dependent progeny. A mating of UM-107 with the *s* strain, 15b, yielded 204 prototrophic to 169 arginine-dependent progeny. The 204 prototrophs were tested for OTC, and were without exception found to have enzyme levels characteristic of the *s* parent. It is concluded that UM-107, derived from wild type, carries a single-gene mutation allelic to *s*, and, by inference, allelic to UM-3. (UM-3 × UM-107 crosses were sterile.) It should be mentioned that crosses of UM-107 or UM-3 to the authors' stock of wild type 73a revealed a genetic factor in the latter which, when combined with the OTC-less mutations, resulted in a genotype which would not grow beyond germination on any medium tested. This factor was not present in any other wild-type strain used, and it is correlated with certain irregularities in arginine metabolism in this 73a stock (DAVIS 1962a) which are now being investigated.

The ratio of OTC-less phenotypes to other citrulline-requiring strains among the mutants isolated indicates that OTC-less mutants are far less frequent when isolated from wild type 74A than when isolated from *s*. A reconstruction experiment involving recovery (by filtration-concentration) of the UM-3 genotype from a mixture of UM-3 and wild-type (74A) conidia indicated good concentration (500– to 2500-fold).

This experiment does not indicate any influence of 74A conidia upon the ultimate isolation of an OTC-less strain. However, the difference between 74A and *s* in their yield of OTC-less mutants may reflect the difference in the amount of OTC in their conidia, which were used as starting materials in filtration-concentration experiments. That is, OTC-less nuclei originating in 74A conidia may be sustained in early growth by residual OTC, and this growth may be sufficient to eliminate them from a successively filtered conidial population growing in minimal medium. In contrast, OTC-less mutants induced in *s* conidia would have only a limited reserve of OTC. Two experiments were done to test this notion. One was the determination of the OTC of wild-type and *s* conidia. The specific activities found (8.1 for wild type and 0.34 for *s*) were, as expected, quite different, and were characteristic of terminal mycelial cultures of the two strains (DAVIS 1962a). However, when conidia of a heterokaryon constituted of UM-107 (OTC^-) and a mutant blocked between citrulline and arginine (OTC^+) were plated on minimal medium, no conidia homokaryotic for UM-107 germinated. Of 148 germlings isolated after 24 and 48 hours, all were heterokaryotic, even though conidia homokaryotic for UM-107 were 7 to 44 percent of the conidial populations

plated. This casts some doubt upon the notion that OTC-less mutants germinate and are filtered out of an irradiated population of OTC$^+$ conidia growing in minimal medium. The foregoing results cannot be used as evidence, however, that the *s* and wild-type alleles of the OTC locus are differentially susceptible to mutation, especially in the light of experiments by WOODWARD (1963) where OTC-less mutants grew for a short time on minimal medium in one of his selection regimes.

DISCUSSION

The data presented clearly define the structural locus for ornithine transcarbamylase in Neurospora. The genetic evidence did not distinguish the mutational site represented by *s* from the UM-3 and UM-107 (OTC-less) mutations. No recombination between *s* and the OTC-less mutant derived from wild type was observed, nor was the *s* phenotype recovered when OTC-less mutants derived from *s* were mated to wild type. The locus is now represented by a number of mutants lacking this enzyme entirely, as well as by the *s* mutant, which has low activity of an OTC with altered kinetic properties (DAVIS 1962b). This locus is distinct genetically and in regard to OTC activity (DAVIS 1962a) from mutants representing the *cit-1* and *cit-2* loci. The structural locus for OTC is here denoted *arg-12* with the descriptive synonym *cit-3*. Allele designations should be the isolation numbers given by those who isolate them. The *s* allele, derived from a single mutational event (HOULAHAN and MITCHELL 1947), will, for consistency, be denoted *s* or *arg-12^s*; the designation *arg-12 s* or *arg-12s* should be avoided because of confusion with double mutants involving the *s* gene.

A point to which attention should be called is that certain mutants, such as OTC-less strains, can be recovered only with difficulty from wild type by the selection procedure used here. Thus, the failure to obtain mutants for a given metabolic step (clearly defined or postulated) cannot be taken as serious evidence for the existence of alternate pathways or enzymes in a pathway. The use of partially deficient mutant strains as starting material for mutant selection may, when available, be as valuable as metabolic inhibitors of the pathway in which mutants are sought.

SUMMARY

Mutants of Neurospora without detectable ornithine transcarbamylase—a class previously undescribed—have been isolated from wild type and from a strain, *s*, having a very low activity for this enzyme. The mutants fall into a single complementation group, and a single locus, allelic to *s*, has been implicated in the specification of the enzyme in wild type. The locus is denoted *arg-12*.

ACKNOWLEDGMENTS

This work has been supported by a grant (G-18012) from the National Science Foundation to the senior author. The technical assistance of MRS. JANE E. ZIMMERMAN is gratefully acknowledged. The senior author thanks DR. VAL W. WOODWARD for discussions of his and our work during the past year.

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