DIRECT INDUCTION IN WILD-TYPE *NEUROSPORA CRASSA* OF MUTANTS (*qa-1^o*) CONSTITUTIVE FOR THE CATABOLISM OF QUINATE AND SHIKIMATE¹

C. W. H. PARTRIDGE, MARY E. CASE² AND NORMAN H. GILES^{2,3}

*Department of Biology, Kline Biology Tower, Yale University,
New Haven, Connecticut 06520*

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ABSTRACT

A color test has been developed for the selection and identification of mutants in *Neurospora crassa*, constitutive for the three normally inducible enzymes which convert quinate to protocatechuate. By this means seven such mutants have been recovered after ultra violet irradiation of wild type and have been shown to be allelic (or very closely linked) to the *qa-1^o* mutants previously obtained by other means. Thus, the regulation of the synthesis of these three catabolic enzymes is indicated to be under the control of a single gene, *qa-1⁺*.

IN *Neurospora crassa* the catabolism of quinic and shikimic acids is controlled by a cluster of four closely linked genes—the *qa* cluster—in linkage group VII closely adjacent to *me-7*. Present evidence indicates that this cluster includes three structural genes encoding the following inducible enzymes: *qa-2*, catabolic dehydroquinase (5-dehydroquinase dehydratase, EC 4.2.1.10), *qa-3*, both quinate dehydrogenase (quinase: NAD oxidoreductase, EC 1.1.1.24) and shikimate dehydrogenase, and *qa-4*, 5-dehydroshikimate dehydrase. In addition, the fourth gene, *qa-1*, apparently produces a regulatory protein which exerts positive control over the synthesis of the three enzymes (VALONE, CASE and GILES 1971).

Previous studies have shown that mutants constitutive for all three of the normally inducible enzymes can be obtained as revertants of certain non-inducible *qa-1* mutants (VALONE, CASE and GILES 1971). These constitutive revertants map very close to the *qa* region and are interpretable as *qa-1^o* mutants.

The recovery of numerous constitutive mutants, all of which are located in the *qa* region and apparently within the *qa-1* gene, supports the view that there is only a single gene in *N. crassa* concerned with regulation of the enzymes encoded in the three structural genes of the *qa* cluster. However, since these constitutive mutants were all selected as revertants of *qa-1* mutants, there appeared to be a possibility that the method of selection might preclude the recovery of constitutive mutants in other genes concerned with regulating the *qa* enzymes. Consequently,

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² Address after July 1, 1972: Department of Zoology, University of Georgia, Athens, Georgia 30601.

³ Author to whom reprint requests should be addressed.

it seemed desirable to attempt to recover constitutive *qa* mutants by testing all survivors after mutagenic treatment of wild type. The present paper discusses a method for detecting such mutants and presents genetic and biochemical evidence that the constitutive mutants recovered to date directly from wild type include only *qa-1^c* types apparently similar to those induced as reversions of *qa-1* mutants.

MATERIALS AND METHODS

In order to obtain *qa* constitutive mutants directly from wild type a method for distinguishing such mutants visually had to be developed. Initially attempts were made to apply the tetrazolium staining method under various conditions, utilizing *qa-1^c* mutants as test strains. However, this approach was finally given up because of high background color in the absence of the specific substrate, quinic acid (QA). This method was only reluctantly abandoned because of reported successes with mutants involving other reducing substrates, both in *Neurospora* (REISS 1967; E. L. TATUM, personal communication), as well as in *Escherichia coli* (LIN, LERNER and JORGENSEN 1962). The latter case involved naked-eye identification of constitutive mutants.

Another approach seemed feasible on the basis of the report (SALAMON and DAVIS 1953) that FeCl_3 produces a strong visible color with protocatechuic acid (PCA) which should be accumulated by any strain of *Neurospora* which has activity for the enzymes leading from QA to PCA provided that QA is supplied and PCA oxygenase is essentially absent or inactive. These requirements would be met by specific constitutivity of the pre-PCA enzymes together with an absence of PCA oxygenase, produced either by employing a PCA oxygenaseless mutant or by exclusion of oxygen from the system. In the absence of such a mutant in *Neurospora*, exclusion of oxygen was employed in the tests.

In practice, the best procedure yet developed is as follows: macroconidia from an albino (*al-2*), pantothenicless (*pan-2*) colonial temperature-sensitive (*cot*) strain (74-OR31-16A, kindly provided by F. J. DE SERRES) were suspended in sterile distilled water after five days growth at 25°, filtered through cotton, centrifuged and resuspended. A volume of 10 ml diluted to an O.D. of 0.6 at 530 nm (approximately 10^7 conidia per ml), was exposed in a rotating quartz flask to a mercury lamp for five minutes at an intensity of 10 ergs/sec/mm² (GILES 1951). After a half hour in the dark, aliquots of 0.05 ml, diluted to contain approximately 300 conidia per aliquot, were spread over the surface of agar plates containing 0.5% tryptose, 0.15% yeast extract, and 0.1% sorbose in Fries minimal medium.

On the following day sterile nylon discs* were pressed into the agar so that reference marks on plates and nylons were superimposed and the plates were incubated for three more days at 25°. After the three-day incubation, the nylons were transferred with sterile forceps to Petri dishes containing Whatman No. 1 filter paper discs moistened with 2.4 ml (per disc) of the following mixture: 200 ml 10% quinic acid (brought to pH 6 with KOH) 100 ml 0.4 M potassium glycinate buffer, pH 9.25, 26 ml 1% FeCl_3 , 2 ml 60 mM NAD, 100 ml dimethylsulfoxide, and water to a final volume of 486 ml. To prevent induction, cycloheximide was included at 30 µg/ml. Within ten minutes the moistened nylons were covered with mineral oil (approximately 10 ml per dish) to exclude oxygen. Those colonies which turned purple during a period of 24 hr were marked by pressing carborundum grains into them. Later, the corresponding colonies were isolated from the agar, allowed to conidiate, and retested in the above manner, using 1 cm spot inocula of heavy conidial suspensions, with an incubation period of two days. Final confirmation of constitutivity was provided by enzyme assays.

Although in experiment A, strain 74A (74-OR23-1A) was used, the albino strain was preferred for avoidance of interfering carotenoid colors in the ferric chloride tests. The dimethyl sulfoxide increased the rate of color development and helped to prevent delayed coloration of wild-type colonies.

* Discs of heavy 60-mesh fabric (National Filter Media Corp., Hamden, Conn., number 154-008-99) were cut with a small soldering iron from pieces which had been swollen by autoclaving under water and kept moist. A guide mark was made on each disc with a felt pen. These discs can be reused after washing in a domestic washer and reautoclaving.

Procedures for growth, extraction, and enzyme assays, were essentially those reported previously (VALONE *et al.* 1971). Specific activities of enzymes are given in nmoles per minute per mg protein at 37°C.

RESULTS

The feasibility of recovering *qa-1^c* mutants in the procedure previously outlined was demonstrated by a reconstruction experiment without mutagenic treatment and by addition of a *qa-1^c* strain (R6-1.5) in a 1:10 ratio to a few plates of irradiated wild type in a mutant hunt. The latter procedure resulted in the recovery of purple colonies from about 50% of the added *qa-1^c* conidia.

In four mutagenic experiments the average apparent rate of induction of mutation to *qa-1^c* from *qa-1⁺* was 7.3×10^{-5} per viable conidium. The albino, *pan*, *cot*, strain was used in all but Experiment A.

The first *qa-1^c* strain obtained by the above procedure was crossed directly to *me-7*. Eight complete asci from the first cross were examined for constitutive ability to convert quinate to protocatechuate and assayed for constitutive thermostable dehydroquinase. One of these ascospore isolates was also assayed for constitutive levels of quinate dehydrogenase, shikimate dehydrogenase, and DHS dehydrase, as well as protocatechuate oxygenase. Certain of the random ascospore isolates of the back-cross of an *F₁* *qa-1^c* to *me-7* were also assayed for constitutive thermostable dehydroquinase after several hundred had been screened by the color tests. All the complete asci showed 1 : 1 segregation of *qa-1^c* and *me-7* by both methods, as indicated in Table 1. From the back cross, 310 *me-7* and 321 *me-7⁺* progeny were screened by the color test. None of the *me-7* strains was *qa-1^c* by this criterion. Seven of the *me-7⁺* strains failed to give a strong positive color test for constitutivity, but were identified as *qa-1^c* by assay for constitutive thermostable dehydroquinase (Table 2). The figures in Table 3 demonstrate that mutant A1-1.4 produced moderately high constitutive levels of all three enzymes under control of the *qa-1* locus.

Similar crosses and tests were performed with the six additional constitutives obtained. The results are summarized in Tables 4 and 5. Four out of 2258 *me-7* progeny tested gave positive color reactions for constitutivity and were subsequently shown to produce thermostable dehydroquinase constitutively. One more of the *F₂* *me-7⁺* progeny among 50 tested failed to show a positive color test, although constitutive thermostable dehydroquinase was detected in its extract. One of the mutants (E4) gave positive color tests among only half of its *F₁* progeny, implying its isolation as a heterokaryon. In summary, no *qa-1⁺* *me-7⁺* recombinant progeny were found among 371 prototrophs tested.

As indicated in Tables 1 and 2, there is considerable variation in the specific activity of constitutive thermostable DHQase among both *F₁* and *F₂* progeny of mutant A1. The *F₁* tetrads of the cross involving mutant A1 and *me-7* were segregating for a morphological variant apparently unlinked to the *qa-1^c* locus. Among these progeny there was a strong correlation between abnormal morphology and low specific activity. Variation in specific activities was also observed among B11 *F₁* progeny and among the recombinant progeny of E6, suggesting segregation of modifying genes affecting enzyme activity levels. The reason for the generally

TABLE 1

*Segregation of qa-1^C and me-7 from a cross of the original qa-1^C isolate A1 with me-7-8a
Seventy-two-hour standing cultures without inducers*

Ascospore Number	C-DHQase Specific activity*	Coloration time (min)†	Growth on minimal medium
1.1	13.0	10	+
1.4	30.0	30	+
1.5	0	—‡	—
1.7	0	—	—
2.1	24.5	30	+
2.4	19.0	10	+
2.5	0	—	—
2.8	0	—	—
3.1	0	—	—
3.4	0	—	—
3.5	7.7	10	+
3.7	25.5	10	+
4.1	0	—	—
4.4	0	—	—
4.5	30.0	10	+
4.7	20.0	10	+
5.1	0	—	—
5.4	0	—	—
5.5	26.0	10	+
5.7	33.0	10	+
6.1	31.0	10	+
6.4	29.0	10	+
6.5	0	—	—
6.7	0	—	—
8.1	0	—	—
8.4	0	—	—
8.5	9.5	210	+
8.7	15.7	210	+
10.1	15.7	10	+
10.4	36.5	30	+
10.5	0	—	—
10.7	0	—	—
qa-1 ^C parent	62		+
WT	0		+

* Thermostable dehydroquinase.

† Time at room temperature required for appearance of definite purple color in colonies on nylon after exposure to quinic acid and ferric chloride (reagent without dimethyl sulfoxide used after freezing colonies).

‡ Dash indicates no purple color produced.

TABLE 2

*Variation in constitutive levels of thermostable DHQase and rates of conversion of quinate to protocatechuate among me-7⁺ random ascospore cultures of F₂ progeny from crosses of qa-1^c isolate A1 with me-7. (Backcross of ascospore 1.4 from first cross)
Seventy-two-hour standing cultures without inducer*

Spore Number	Coloration time (hrs)*	Specific activity†
19	—‡	1.3
85	—	5.2
110	72	4.8
115	—	2.6
144	72	14.8
242	24	54.0
251	1	21.5
WT	—	0

* Time at room temperature required for appearance of definite purple color in colonies on nylon after exposure to quinic acid and ferric chloride.

† Thermostable dehydroquinase.

‡ Dash indicates no purple color produced.

poor correlation between the rate of color development and the level of thermostable DHQase among these strains is unclear. However, it has been noted (VALONE, CASE and GILES 1971; R. CHALEFF, unpublished) that the enzymes required for the color reaction are less closely coordinated in *qa-1^c* strains than in *qa-1⁺* strains, where the coordinancy of induction seems to be complete. The figures in Table 3 do not show much deviation from coordinancy in the case of mutant A1-1.4 grown at two different temperatures.

DISCUSSION

The color test adopted for detection of mutants constitutive for the enzymes converting quinic acid (or shikimic acid) to protocatechuic acid has proven to be reasonably efficient. At the mutant hunt stage, reconstruction experiments indicate that a considerable fraction of such mutants may pass undetected. However, one of the new mutants obtained (E4) was apparently isolated as a heterocaryon, indicating that the method is not limited to detection of colonies arising as pure clones. This result was not unexpected in view of the previous indication of domi-

TABLE 3

*Assay of degradative enzymes of qa-1^c ascospore isolate A1-1.4 after
24-hour shake culture without inducer**

Growth temp. (°C)	QDH	SDH	Specific activities of: DHQase (thermostable)	DHS dehydrase	PCA oxygenase
25	127	380	97	33	17
35	106	287	106	34	30

* Wild-type levels were routinely found to be less than 1% of these values (VALONE *et al.* 1971).

TABLE 4

Summary of tests for recombinants in crosses of constitutive mutants $\times$ me-7

Experiment Number	A	B	E	E	E	E	G	Total
Isolate number	1	11	1	2	4	6	2	
F_1^*								
Number of progeny tested	16	9	10	6	15	5	18	
Number of $qa-1^c$ progeny	16†	9†	10	6	8	5	18	
F_2								
$qa-1^c$ parent	A1-1.1A	B11-3a	E1-5A	E1-1A	E2-3A	E4-3A	E6-3A	G2-4A
Number of $me-7$								
progeny tested	310	241	273	306	285	278	289	2258
Number of $qa-1^c$ progeny	0	0	0	2‡	0	0	2‡	4
Number of $me-7^+$								
progeny tested	321	17	9			6	18	371
Number of $qa-1^c$								
progeny by color test	318	17	9			6	17	367
corrected‡	321	17	9			6	18	371

* Only prototrophs tested.

† Demonstrated by dehydroquinase assay.

‡ Demonstrated by dehydroquinase assay of progeny giving negative color test.

nance of $qa-1^c$ (VALONE, CASE and GILES 1971). As a tool for measuring linkage (e.g. to $me-7$) the color test method was sufficient to detect all but a small minority of the non-recombinant progeny (ca. 1% in the case of mutant A1). Among the apparent $qa-1^c$ $me-7$ recombinant progeny indicated by the color test none failed to be confirmed by enzyme assay. The linkage indicated in this way between the constitutive mutations induced in wild type (as a whole) and $me-7$ (ca. 0.2% recombination) is not significantly different from that previously established for $qa-1^c$ mutations induced as revertants $qa-1$ strains. Thus, there is no evidence for more than one regulatory gene for the synthesis of the enzymes degrading quinic acid to protocatechuic acid, although quite different selection methods have been employed in searching for mutants affecting the regulation of quinate catabolism.

Before the completion of this investigation a report was published by INGLEDEW, TRESGUERRES and CANOVAS (1971) on a related study of a bacterium. These

TABLE 5

Constitutive levels of thermostable DHQase and rates of conversion of quinate to protocatechuate for apparent recombinants among random ascospore cultures of F_2 progeny from crosses of $qa-1^c$ isolates with $me-7$

Strain	Growth requirement	Purple coloration rate	Specific activity
E6-3-418	met	rapid	11
E6-3-200	met	rapid	27
E1-1-499	met	slow	12
E1-1-468	met	slow	16
E6-3-14	none	nil	19

workers were able to observe mutants deficient in protocatechuate oxygenase directly on agar plates during growth by inclusion of ferric iron in the medium and subsequently to detect mutations which interfered with the conversion of quinic acid to protocatechuic acid, including those defective in regulatory functions. We were unable to obtain as good a color reaction with this kind of treatment of *Neurospora* as in the method given in this paper. However, it was possible to obtain moderately strong purple coloration of *qa-1^c* colonies (or pre-induced *qa-1⁺* colonies) directly on the agar by overplating, after growth, with an equal volume of a mixture similar to that used with the nylon replicas, but containing 0.75% agar and enough pH 10 buffer to bring the final average pH above 9. The colonies remained viable under these conditions for at least several hours. No oil was required to maintain color for a period of days in the agar.

After this investigation was initiated, additional studies (CASE and GILES, unpublished) were undertaken with the original *qa-1* mutants isolated by RINES (1968) utilizing the filtration-concentration technique. Growth tests and enzyme assays established that two of these mutants can be classified as temperature-sensitive constitutives as well as temperature-sensitive mutants—acting as mutants at 25° and as constitutives at 35°. Furthermore, recombination studies have established that such constitutives map within the *qa-1* gene. These results provide additional evidence for the direct recovery from wild type of *qa-1^c* mutant types.

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