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Characterization of *qa-2* Mutants of *Neurospora crassa* by Genetic, Enzymatic, and Immunological Techniques

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Genetic and complementation mapping studies using 20 *qa-2* mutants defective for catabolic dehydroquinase indicate that the *qa-2* gene encodes a single polypeptide chain and is the structural gene for catabolic dehydroquinase, a 220,000-molecular-weight protein composed of identical 10,000-molecular-weight subunits. Many *qa-2* mutants are capable of reversion, but no evidence has yet been obtained for nonsense mutations in this gene. The biochemical consequences of the mutations in two complementing *qa-2* strains (M239 and M204) have been determined. Both mutants have extremely low levels of catalytic activity and form a heterocaryon with about 4% of the wild-type activity. As assayed by immunological cross-reactivity, mutant M239 and the heterocaryon have nearly wild-type levels of native-molecular-weight catabolic dehydroquinase protein, whereas M204 has no detectable amount of this protein. Thus it is concluded that M239 has a mutation at or near the catalytic site which reduces the activity 10,000-fold but has little or no influence on the formation of the native multimeric structure. In contrast, M204 apparently has a mutation that severely inhibits aggregation and may have only a minor effect on the inherent potential for catalytic conversion at the reactive site. The heterocaryon would appear to form a mixed multimer with the monomeric subunits from M239 providing the aggregated structure and those from M204, the catalytically active moiety.

In *Neurospora crassa*, the three reactions in the catabolism of quinic acid to protocatechuic acid are controlled by a tightly linked cluster of four genes, the *qa* cluster. Three of these genes are the structural genes for the three enzymes catalyzing the three reactions. *qa-2* encodes catabolic dehydroquinase (5-dehydroquinate hydrolyase, EC 4.2.1.10); *qa-3* encodes quinate dehydrogenase (quinate:NAD^+ oxidoreductase, EC 1.1.1.24); and *qa-4* encodes dehydroshikimate dehydrase. The fourth gene, *qa-1*, encodes a regulatory protein that, in the presence of inducer (quinic acid), acts in a positive fashion to initiate coordinate synthesis of the three *qa* enzymes (3-6). Evidence supporting this hypothesis has come from comparative studies of purified catabolic dehydroquinase (7) obtained from the wild type and from mutants bearing lesions in the *qa* gene cluster (9). These studies indicate that the properties of purified catabolic dehydroquinase obtained from the wild type and from mutants with lesions in the *qa-1* gene or the *qa-3* gene are identical, as predicated by the hypothesis. In contrast, the catabolic dehydroquinase from one *qa-2* mutant (M237) was found to be 1,500-fold less active and appeared to have a slightly different amino acid content (9). Despite the low activity, this mutant was shown to be immunologically competent in tests with antisera against catabolic dehydroquinase and to have wild-type levels of catabolic dehydroquinase protein. These results are interpreted as indicative of an alteration in strain M237 at or near the catalytic site of the enzyme that has little or no influence on the native structure of the molecule.

The present studies were undertaken to characterize additional *qa-2* mutants genetically, enzymatically, and immunologically. Such studies promised to be of particular interest because of the unusual properties of catabolic dehydroquinase, including the relative ease with which it can be purified. The purified native enzyme is extremely resistant to thermal and chemical denaturation, has a very high specific activity, and, with a molecular weight of approximately 220,000, is composed of about 20 identical subunits each with an approximate molecular weight of 10,000 (7). The structure of the native enzyme suggested that mutants might well be obtained that would affect aggregation. In addition, allelic complementation had already been reported to occur between *qa-2* mutants (H. W. Rines, Ph.D. thesis, Yale

University, New Haven, Conn., 1969), but the properties of the resulting active hybrid enzyme had been only partially characterized.

MATERIALS AND METHODS

Strains. The wild-type strain 74A and a *qa-3* mutant M16 were used as sources of wild-type *qa-2* gene product. The *qa-2* mutant strains were isolated and first characterized by Rines (Rines, Ph.D. thesis, 1969) who used a single *arom-9* strain (which lacks the biosynthetic dehydroquinase) derived from 74A. Such double mutants (*qa-2 arom-9*) have a multiple aromatic requirement. All *qa-2* strains studied were crossed to the complementing *pan-2* allele B36 to obtain genetically marked double *qa-2 pan-2* mutants for use in crossing tests. In addition, one *qa-2* strain (M204) was crossed to the complementing *pan-2* allele B23. This *qa-2* (M204) *pan-2* (B23) strain was used to force a complementing heterocaryon with the *qa-2* (M239) *pan-2* (B36) strain for use in the enzyme extraction studies.

Genetic analyses. To obtain a fine-structure genetic map of the *qa-2* gene, crosses were made of all pairwise combinations of *qa-2 pan-2* (B36) strains on Difco corn meal agar supplemented with calcium panthothenate (2 mg/liter). The *pan-2* marker served to detect possible contaminants. The ascospores from each cross were suspended in distilled water and heat shocked for 1 h at 60°C. The ascospore suspension from each cross was then added to 400 ml of Fries liquid minimal medium supplemented with calcium panthothenate (2 mg/liter) and 0.3% quinic acid (as a sole carbon source). Portions were dispensed at a concentration of about 4,000 spores per 3 ml of medium in test tubes (13 by 100 mm). (Ascopore plating into agar medium could not be used since *qa-2* mutants grow appreciably even in washed agar.) Prototrophs were detected as growth in individual tubes on quinic acid. At the spore concentration used, no more than one prototroph per tube would be expected. Therefore, the map distance is based on the percentage of prototrophs in the total number of ascospores analyzed for each cross. The total number of ascospores was determined by counting a sample of the ascospore suspension from each cross before dispensing into the tubes. Since *qa-2* mutants essentially fail to exhibit allelic complementation on quinic acid medium, pseudo-wild types are not recovered in crossing analyses performed in the manner described.

Complementation tests. Allelic complementation tests were performed at both 25 and 35°C. Mixed conidial suspensions using all pairwise combinations of *qa-2 arom-9* mutants were inoculated into Fries liquid minimal sucrose medium, and the tubes were examined for growth at suitable intervals. Control inoculations were performed to test for the stability of parental strains.

Reversion analyses. All *qa-2* mutants were tested for their ability to revert both spontaneously and after ultraviolet irradiation. For this purpose, *qa-2 arom-9* double mutants were used, and conidia were plated on Fries minimal sucrose medium. Revertants were isolated and tested for their ability to grow on quinic acid as a carbon source.

Tests for nonsense suppressors. To determine whether selected *qa-2* mutants were suppressible, conidia from double mutants of eight noncomplementing and three complementing mutants combined with *arom* mutant 54, which has been shown to carry a suppressible nonsense mutation (1), were irradiated with ultraviolet light and plated on Fries sucrose medium alone or supplemented with 80 mg of both phenylalanine and tyrosine per liter. Revertants lacking an aromatic requirement were isolated and tested for their ability to grow on quinic acid as a carbon source. Growth on quinic acid would indicate the simultaneous suppression of both *arom-54* and of a particular *qa-2* mutation.

Growth conditions. For preparative-scale enzyme purification, strains M204 and M239 were grown and induced in 200-liter cultures as previously described (7). For small-scale enzyme extractions, cultures of all strains included in Table 2 were started by conidial inoculation of six 2,000-ml Erlenmeyer flasks containing 500 ml of Fries minimal medium plus 1.5% sucrose and any required supplement. The cultures were grown at 25°C for 24 h at 300 rpm on a rotary shaker. The mycelia were harvested by filtration, washed with water, and induced by shaking in six 2,000-ml Erlenmeyer flasks containing 500 ml of Fries minimal medium plus 0.3% quinic acid and any required supplement for 6 h at 25°C. The mycelia were harvested by filtration, washed with water, and frozen at −70°C until used. The only strains requiring supplementation were M237, M239, and M204, which were grown and induced in the presence of 2 mg of calcium pantothenate per liter.

Enzyme extraction and assay. Catabolic dehydroquinase was purified according to the method previously described (7). The enzymatic and immunological comparisons of the various strains and the heterocaryon were performed on partially purified samples which had only been subjected to the 71°C heat step, ammonium sulfate fractionation, and dialysis into 0.01 M potassium phosphate, pH 7.5, plus 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM ethylenediaminetetraacetic acid. The assay used for catabolic dehydroquinase has been reported previously (3). One unit of activity is defined as the production of 1 nmol of dehydroshikimate per min at 37°C.

Protein determination. Protein concentrations were determined by the micro-biuret technique (8).

Sucrose density gradient centrifugation. Sucrose density gradient centrifugations were performed according to the method of Martin and Ames (11) at 37,000 rpm in an SW-65 rotor at 4°C for 18 h, using Ultra-Pure grade sucrose from Schwarz/Mann. Catalase (molecular weight, 240,000) and alkaline phosphatase (molecular weight, 86,000) were used as internal standard proteins for the determination of the native molecular weight; cytochrome *c* (molecular weight, 12,400) was used in the subunit molecular weight measurements.

Immunology. Antisera against pure catabolic dehydroquinase from strain M16 were produced in New Zealand white rabbits by standard injection, bleeding, and serum separation techniques. Immunological cross-reactivity was assayed by the Ouch-

terlony double-immunodiffusion technique (12) and by the Mancini single-radial-diffusion method (10).

RESULTS

Genetic and complementation maps. The fine-structure genetic map derived from the crossing analyses is indicated in Fig. 1. The relative positioning of mutations on the genetic map is based on the prototroph frequency for each cross. A minimum of nine separable mutational sites within the *qa-2* gene have been detected. The cluster of mutations located at the right end of the genetic map have not been separated because considerably larger numbers of ascospores would have to be analyzed than have been done to date. For example, the mutational sites in M246 and M247, which are located at the left end of the genetic map (Fig. 1), were separated only when more than 10^6 ascospores were tested. Mutants M201 and M251 appear to be multiple-site (deletion) mutants. These results, however, need to be confirmed by additional crosses since mutant M201 is revertible (Table 1).

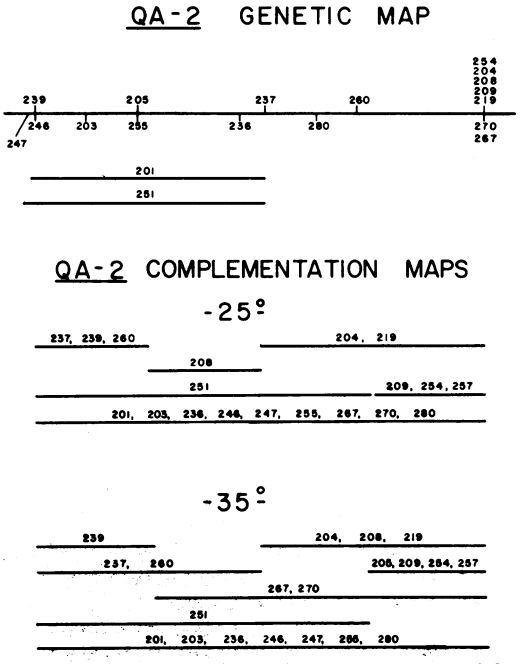

FIG. 1. *Genetic and complementation maps of the qa-2 gene. For details of mapping procedures, see Materials and Methods. The lesion in mutant M257 has not been positioned on the genetic map. Mutant M205 is too leaky at 25°C for use in complementation tests. Based on percentage of prototrophs, the map distance between the mutations in strains M246 and M254 is 0.00047. On the genetic map, the lesions in complementing mutants are placed above the line, and those in noncomplementing, below.*

The complementation maps based on tests at both 25 and 35°C are also indicated in Fig. 1. In general the two maps are quite similar, although certain minor temperature-dependent differences are evident. Also, there is a general co-linearity between the complementation maps and the genetic map.

Reversion analyses. The ability of *qa-2* strains to revert, either spontaneously or after ultraviolet treatment, was tested by using *qa-2 arom-9* double mutants (Table 1). Mutants M201, M205, and M208 were too leaky to determine a spontaneous reversion rate. Five of eight complementing mutants reverted, as did about half of the noncomplementing mutants. Although many of the revertants are presumably due to true back-mutations, the possibility remained open that some might contain suppressor mutations, especially revertants induced in noncomplementing mutants.

Attempts to detect nonsense suppressors involved the use of *qa-2 arom 54* double mutants as described in Materials and Methods. To date, extensive tests of eight noncomplementing and three complementing *qa-2* mutants have failed to produce any evidence for nonsense suppressors affecting *qa-2* mutants since none of the revertants obtained were able to grow on quinic acid.

TABLE 1. *Results of reversion analyses of the qa-2 mutations in qa-2 arom-9 strains and qa-2 arom 54 strains*

Mutant type	Stable	Revertible[a]
qa-2 arom-9		
Noncomplementing	M236, M246, M247	M203, M255, M267, M280
Complementing	M204, M239, M260	M209, M219, M237 M254, M257, M270
Leaky	M251	M201, M205, M208
qa-2 arom 54		
Noncomplementing	M236, M246, M247, M201 M203, M255, M267, M280	
Complementing	M237, M254, M270	

[a] Either spontaneously or after ultraviolet treatment.

TABLE 2. *Levels of catabolic dehydroquinase activity and native multimeric protein in the dialyzed 0 to 33% ammonium sulfate fractions from several strains of Neurospora crassa*

Strain	Activity (U[a]/ml)	Mancini ring diam (mm)	Native protein (μg/ml)	Sp act (U/mg)
74A	19.3	2.5	100	193
M16	53.8	5.5	225	239
M237	0.026	4.5	160	0.16
M239	0.002	2.0	80	0.02
M204	0.008	0.0	<2[b]	>4.0
M204 + M239 heterocaryon	0.71	2.0	80	8.9

[a] Nanomoles of dehydroshikimate produced per minute at 37°C.

[b] The limit of detection by the Mancini assay is about 2 μg/ml.

Biochemical consequences of the mutations in two complementing *qa-2* alleles and their interaction in a heterocaryon. For this analysis, the levels of enzymatic activity and of immunologically competent native catabolic dehydroquinase protein were determined in the dialyzed 0 to 33% ammonium sulfate fraction of *qa-2* mutants M204 and M239 and their heterocaryon and in strains 74A, M16, and M237 for purposes of comparison (Table 2). The enzymatic activity present in both of the *qa-2* mutants and the heterocaryon appears to correspond to the native 220,000-molecular-weight multimer. The heterocaryotic activity cosediments with the wild-type activity in sucrose density gradients (Fig. 2), as does the activity present in both M204 and M239. The catabolic dehydroquinase activity present in each of the two *qa-2* mutants was carried through the normal purification procedure and found to behave identically to the wild-type multimeric protein on both G-200 and diethylaminoethyl-cellulose chromatography (7). The immunological cross-reacting species assayed by the Mancini single-radial-diffusion technique was shown to be identical to native catabolic dehydroquinase protein by immunological homology with purified enzyme on Ouchterlony plates (Fig. 3) and cosedimentation of the cross-reactivity with catalytic activity in sucrose density gradients. The concentration of immunologically competent catabolic dehydroquinase protein was determined from the diameter of the Mancini precipitin ring by application of the standard curve shown in Fig. 4. The same dilution of a single sample of antisera was used for the Mancini assay of the standards and the unknowns.

The most striking aspect of these data is that both the M239 mutant strain and the M204 + M239 heterocaryon produce nearly wild-type (74A) levels of multimeric catabolic dehydroquinase protein. However, the specific activity is reduced nearly 10,000-fold in strain M239 and

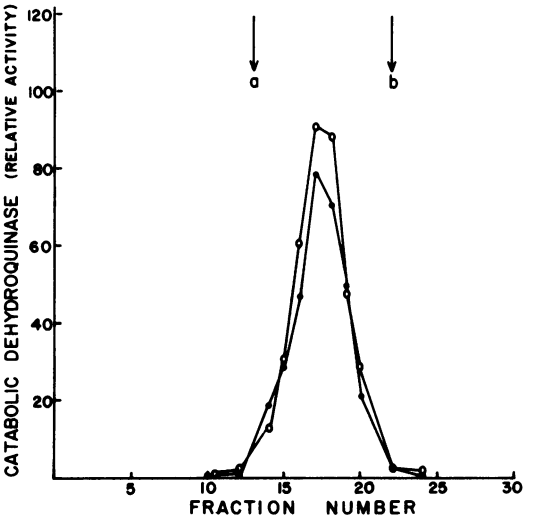

FIG. 2. *Sucrose density gradient centrifugation of catabolic dehydroquinase activity contained in the dialyzed 0 to 33% ammonium sulfate fraction of the qa-3 mutant M16 (○) and the M204 + M239 heterocaryon (●). Internal standards were catalase (a) and alkaline phosphatase (b).*

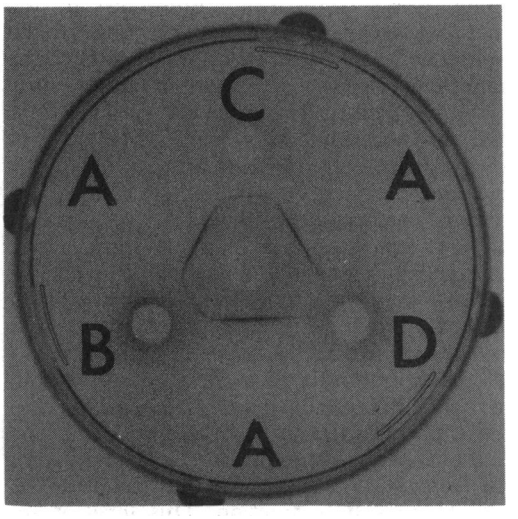

FIG. 3. *Ouchterlony double-immunodiffusion plate showing the homology of purified catabolic dehydroquinase from M16 (A) with the cross-reacting species in the 0 to 33% ammonium sulfate fraction from strain M239 (B) and the heterocaryon (C). This species is absent in the 0 to 33% ammonium sulfate fraction from strain M204 (D).*

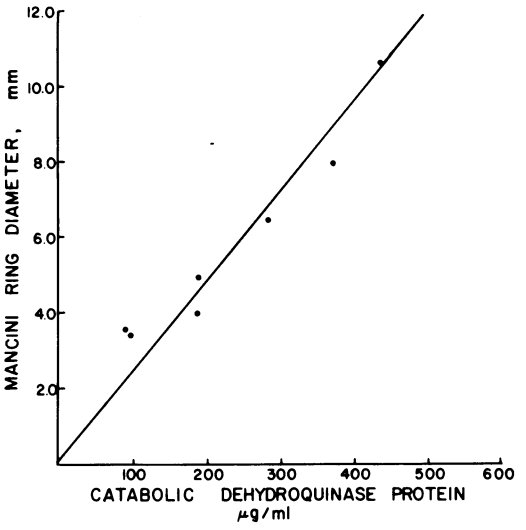

FIG. 4. *Standard curve relating concentration of native catabolic dehydroquinase protein to diameter of Mancini immunoprecipitin ring. Pure samples of catabolic dehydroquinase from strain M16 were used for construction of this curve and absolute protein concentrations were determined by the micro-biuret technique.*

only approximately 25-fold in the heterocaryon. In contrast, strain M204 produces no detectable amount of native catabolic dehydroquinase protein, even though it has a slightly higher catalytic activity than strain M239.

This large quantitative difference in the amount of native *qa-2* protein produced by the two mutants was verified by the results of the preparative-scale purification of the activity from each strain. The catalytic activity (0.17 total unit) purified from strain M204 represents an extremely low level of catabolic dehydroquinase protein. No immunological cross-reaction corresponding to the native enzyme can be detected, and the electrophoretic gels of the purified material are virtually clear. In contrast, the catalytic activity (0.09 total unit) purified from strain M239 represents a high level of protein which has a strong immunological cross-reaction for the native protein and an electrophoretic band pattern very similar to the wild type in both intensity and position. The yield of purified catabolic dehydroquinase protein from M239 is 73% based on the 0 to 33% ammonium sulfate fraction. This level of enzyme recovery is quite comparable to that obtained in the purification from the wild type.

Presence of the catabolic dehydroquinase subunit in extracts of *qa-2* mutant M204. Since strain M204 produces only low levels of native catabolic dehydroquinase protein and yet complements well with strain M239 to produce a large increase in enzymatic activity, we examined the level of monomeric subunit *qa-2* protein in this strain after induction. Antiserum produced against purified catabolic dehydroquinase apparently has a weak, but measurable, cross-reaction with the monomeric subunit. That this second cross-reacting species is the subunit has been supported by its sedimentation behavior in sucrose density gradients, its Stokes radius as determined by gel filtration, and the absence of tryptophan (J. A. Hautala, unpublished data). It also has the same amino terminal amino acid (proline) as the native enzyme (Per Strøman, unpublished data). The degree of immunological cross-reaction with the antiserum is proportional to the subunit concentration. However, the level of cross-reaction cannot be correlated with absolute protein concentration due to the extremely small amounts of subunit protein that can be obtained in a pure form.

As assayed by immunological cross-reactivity, strain M204 does not produce elevated levels of the *qa-2* monomeric subunit that can be extracted. The 0 to 33% ammonium sulfate fraction contains the subunit at concentrations comparable to that found in 74A, M16, M237, M239, and the M204 + M239 heterocaryon. No additional subunit protein is present in any other ammonium sulfate fraction up to 75% saturation. The level of the subunit cross-reacting material is also the same in unheated as in heated samples.

DISCUSSION

The genetic and complementation mapping studies with *qa-2* mutants provide evidence that the *qa-2* gene encodes a single polypeptide. This evidence comes from the relatively frequent occurrence of noncomplementing *qa-2* mutations and the fact that these are located at various sites throughout the *qa-2* gene. Also, the pattern of complementation is one that is characteristic of allelic mutants. Independent data support the additional conclusion that the *qa-2* gene is the structural gene for catabolic dehydroquinase. Rines (Ph.D. thesis, 1969) obtained evidence that the active catabolic dehydroquinase formed in a heterocaryon between two complementing mutants (M204 and M239) is more thermolabile than the wild-type enzyme. Furthermore, comparative amino acid analyses of purified catabolic dehydroquinase protein from the wild-type and the *qa-2* mutant M237 suggest that M237 protein differs from the wild type by a single amino acid substitution (9).

The studies of revertants suggest that many *qa-2* mutants probably differ from the wild type by single base pair alterations. The possibility cannot be rigorously excluded that some revertants are the result of suppressor mutations. However, attempts to detect suppressors of various *qa-2* mutations, utilizing reversion tests of appropriate *qa-2 arom 54* strains, have been unsuccessful.

The data presented in Table 2 on the relative levels of catabolic dehydroquinase activity and native multimeric protein for strains M204 and M239 provide information on the nature of the individual mutational alterations. It would appear that the enzyme in strain M239 is altered at or near the catalytic site. This alteration reduces the enzymatic activity by a factor of 10,000 but has little or no influence on the formation of the native aggregated structure. The biochemical result of the mutation in M239 is thus quite similar to that in M237. In contrast, M204 produces very little native multimeric protein, but that which is produced has a much higher specific activity than that from M239. The mutational alteration present in M204 appears to inhibit strongly the formation of the native multimeric structure but may have little effect on the inherent potential for catalytic conversion at the reactive site. Preliminary experiments have shown that another complementing mutant (M209) in which the lesion has been mapped very near to that in M204 also produces no detectable level of native catabolic dehydroquinase protein on induction.

When M239 and M204 interact to form a heterocaryon, the level of native catabolic dehydroquinase protein is virtually identical to that in the M239 parent alone, but the specific activity is increased by a factor of approximately 450. Thus, it would appear that in the heterocaryon a mixed multimer is formed with the monomeric subunits from the M239 parent providing the aggregated structure, and those from M204, the enzymatically reactive moiety.

Since strain M204 forms exceedingly low levels of the native catabolic dehydroquinase multimer, the absence of an elevated level of the monomeric subunit in this strain is somewhat surprising, particularly in view of its complementation behavior. One possible explanation is that in the absence of aggregation the subunit is very labile and is destroyed during extraction or rapidly degraded in vivo such that only a low-level pool is maintained. When the heterocaryon is formed, the aggregation competent M239 protein may protect the M204 monomers from either in vitro or in vivo degradation by incorporating these monomers into a stable catabolic dehydroquinase multimer. A second hypothesis is that strain M204 produces only a low level of monomer but that the potential catalytic activity of these monomers is at the wild-type level. Since the incorporation of only one such M204 monomer with approximately 19 M239 monomers could produce a heterocaryotic multimer with the level of catalytic activity observed (Table 2), even a low level of M204 monomer could account for the complementation observed. Testing of this hypothesis requires a method for determining the relative numbers of M204 and M239 monomers in the heterocaryotic multimeric protein. In vitro complementation using a radioactively labeled monomer from one of the strains would serve this purpose, but to date we have been unable to devise a successful technique for achieving effective in vitro complementation. This hypothesis also requires that strain M204 have a lesion in a regulatory region at one end of the *qa-2* gene. We have as yet no evidence that such a region exists. A third possible explanation is that the M204 subunit has a lower level of cross-reactivity and therefore cannot be assayed accurately by immunological techniques.

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LITERATURE CITED

1. **Case, M. E., and N. H. Giles.** 1968. Evidence for nonsense mutations in the *arom* gene cluster of *Neurospora crassa*. Genetics 60:49–58.
2. **Case, M. E., and N. H. Giles.** 1975. Genetic evidence on the organization and action of the *qa-1* gene product: a protein regulating the induction of three enzymes in quinate catabolism in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S.A. 72:553–557.
3. **Case, M. E., N. H. Giles, and C. H. Doy.** 1972. Genetical and biochemical evidence for further interrelationships between the polyaromatic synthetic and the quinate-shikimate catabolic pathways in *Neurospora crassa*. Genetics 71:337–348.
4. **Chaleff, R. S.** 1974. The inducible quinate-shikimate catabolic pathway in *Neurospora crassa*: genetic organization. J. Gen. Microbiol. 81:337–355.
5. **Chaleff, R. S.** 1974. The inducible quinate-shikimate catabolic pathway in *Neurospora crassa*: induction and regulation of enzyme synthesis. J. Gen. Microbiol. 81:357–372.
6. **Giles, N. H., M. E. Case, and J. W. Jacobson.** 1973. Genetic regulation of quinate-shikimate catabolism in *Neurospora crassa*, p. 309–314. *In* B. A. Hamkalo and J. Papaconstantinou (ed.), Molecular cytogenetics. Plenum Press, New York.
7. **Hautala, J. A., J. W. Jacobson, M. E. Case, and N. H.**

Giles. 1975. Purification and characterization of catabolic dehydroquinase, an enzyme in the inducible quinic acid catabolic pathway of *Neurospora crassa*. J. Biol. Chem. 250:6008–6014.

8. **Itzbaki, R. F., and D. M. Gill.** 1964. A micro-biuret method for estimating proteins. Anal. Biochem. 9:401–410.
9. **Jacobson, J. W., J. A. Hautala, M. E. Case, and N. H. Giles.** 1975. Effect of mutations in the *qa* gene cluster of *Neurospora crassa* on the enzyme catabolic dehydroquinase. J. Bacteriol. 124:491–496.
10. **Mancini, G., A. O. Carbonara, and J. F. Heremans.** 1965. Immunochemical quantitation of antigens. Immunochemistry 2:235–254.
11. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.
12. **Ouchterlony, Ö.** 1967. Immunodiffusion and immunoelectrophoresis, p. 655–706. *In* D. M. Wier (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, England.