Genetic Regulation of the Quinic Acid Utilization (*QUT*) Gene Cluster in *Aspergillus nidulans*

By SUSAN GRANT,¹ C. F. ROBERTS,¹ HEATHER LAMB², M. STOUT²
AND A. R. HAWKINS^{2*}

¹Department of Genetics, The University, Leicester LE1 7RH, UK

²Department of Genetics, The University, Newcastle upon Tyne NE1 7RU, UK

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A large number of quinic acid non-utilizing *qut* mutants of *Aspergillus nidulans* deficient in the induction of all three quinic acid specific enzymes have been analysed. One class, the *qutD* mutants, are all recessive and are non-inducible at pH 6.5 due to inferred deficiency in a quinate ion permease. Two regulatory genes have been identified. The *QUTA* gene encodes an activator protein since most *qutA* mutants are recessive and non-inducible although a few fully dominant mutants have been found. The *QUTR* gene encodes a repressor protein since recessive mutations are constitutive for all three enzyme activities. Rare dominant non-inducible mutants which revert readily to yield a high proportion of constitutive strains are inferred to be *qutR* mutants defective in binding the inducer. The gene cluster has been mapped in the right arm of chromosome VIII in the order: centromere – > 50 map units – *ornB* – 12 map units – *qutC* (dehydratase) – 0.8 map units – *qutD* (permease), *qutB* (dehydrogenase), *qutE* (dehydroquinase), *qutA* (activator) – 4.4 map units – *qutR* (repressor) – 20 map units – *galG*. This organization differs from that of the *qa* gene cluster in *Neurospora crassa*, particularly in the displacement of *qutC* and *qutR*.

INTRODUCTION

The initial stages in the degradation of quinic acid by *Aspergillus nidulans* and other fungi include two metabolic intermediates, 3-dehydroquinic acid and dehydroshikimic acid, which are also produced in the common pathway for the synthesis of aromatic amino acids. Two distinct isoenzymes interconvert these substrates and are encoded by two genes subject to different genetic and physiological controls (Hawkins *et al.*, 1982; Kinghorn & Hawkins, 1982; Giles *et al.*, 1967*b*). One isoenzyme, biosynthetic 3-dehydroquinase, is a component of a pentafunctional polypeptide encoded by a single complex locus which is expressed at low constitutive levels (Charles *et al.*, 1986; Giles *et al.*, 1967*a*). The other isoenzyme, catabolic 3-dehydroquinase, is a single-function polypeptide encoded by a gene which is a member of a cluster of tightly linked structural genes highly inducible by quinic acid and under the control of linked regulatory genes (Hawkins *et al.*, 1982, 1984; Case & Giles, 1975).

We wish to understand the origin of these different genetic functions in *A. nidulans*, and the genetic and molecular mechanisms which regulate and co-ordinate their expression and maintain physiological balance between potentially competing metabolic pathways. The *AROM* locus encoding the pentafunctional polypeptide has been isolated and sequenced (Kinghorn & Hawkins, 1982; Charles *et al.*, 1985, 1986), and strong homology has been found with the corresponding bacterial genes, suggesting common phylogenetic origin and multiple gene fusions (Hawkins, 1987).

Quinic acid is a good carbon source for *A. nidulans*, inducing high levels of the three enzymes

Abbreviations: PCA, protocatechuic acid; RF, recombination frequency.

specific for degradation to protocatechuic acid, which are encoded by three closely linked genes *QUTB*, dehydrogenase (EC 1.1.1.24) (1), *QUTE*, catabolic 3-dehydroquinase (EC 4.2.1.10) (2) and *QUTC*, dehydratase (3). Mutations in these genes cause the loss of a single enzyme activity, and a second class of closely linked pleiotropic point mutations result in the failure to induce all three enzyme activities (Hawkins *et al.*, 1982). The non-inducible mutants generally complement single enzyme deficient mutants in heterozygous diploids, indicating their potential to produce all three enzyme activities and *trans* activity of the normal gene product (Hawkins *et al.*, 1984). Molecular analysis of the gene cluster has been initiated by the isolation of 13.2 kb *A. nidulans* genomic DNA in recombinant phage λ -Q1 using the catabolic 3-dehydroquinase gene *QA-2* of *Neurospora crassa* as a hybridization probe (Hawkins *et al.*, 1985).

Previous analysis of the *QA* system in *N. crassa* (see Giles *et al.*, 1985, for a recent review) has shown that three tightly linked structural genes *QA-2* (2), *QA-4* (3) and *QA-3* (1) are regulated by an adjacent region *QA-1*, in which two classes of non-inducible mutants producing slow (*qa-1S*) or fast (*qa-1F*) complementation in heterokaryons are separately located. Molecular analysis has revealed two genes in the *QA-1* region (Huet, 1984), which are interpreted to encode activator (*QA-1F*) and repressor (*QA-1S*) proteins, and also two further quinic acid induced genes *QA-X* and *QA-Y* of unknown function (Patel *et al.*, 1981).

This paper describes the isolation and analysis of a large number of non-inducible *qut* mutants in *A. nidulans*. The majority are at the *QUTA* locus and their various properties indicate that the *QUTA* gene encodes an activator protein. The isolation of constitutive mutants and their distinct properties identify a new gene *QUTR* encoding a repressor protein, and linked (RF = 4.4%) to the *QUT* gene cluster.

METHODS

Strains, media, and genetic and biochemical techniques. These were as previously described (Hawkins *et al.*, 1984).

Qualitative test for the overall reaction from quinic acid to protocatechuic acid (PCA). This was developed from the procedure described by Partridge *et al.* (1972). The freshly prepared reaction mixture contained 5.0 ml dimethyl sulphoxide (Spectrosol, BDH); 5.0 ml 0.4 M-potassium glycinate pH 9.2; 5.0 ml 20% (w/v) quinic acid pH 6.5; 1.3 ml 1% (w/v) FeCl_3 ; 1.0 ml cycloheximide (3 mg ml⁻¹ solution); 7.0 ml distilled water; 5.0 mg NAD.

Samples of mycelium in 4 mm³ blocks of agar (see below) were placed in 1 × 6 cm disposable plastic tubes and 0.75 ml reaction mixture was added. Paraffin oil was overlaid to exclude air and the tubes were incubated at 30 °C overnight. Mycelium is permeabilized by dimethyl sulphoxide, particularly to NAD cofactor for the dehydrogenase, and PCA produced from quinic acid by the overall three enzyme activities is detected as an intense red colouration developed by complex formation with Fe^{3+} . Atmospheric oxygen is excluded to prevent the further metabolism of PCA by oxygenase, although in the procedure described it was unnecessary and equivalent results were obtained with or without the addition of paraffin oil.

Samples of mycelium were grown from a heavy inoculum of conidiospores placed as a patch 1 cm diameter on the surface of minimal agar and incubated overnight at 37 °C. Mycelium should be taken in primary growth and before any sign of conidiation, when enzyme levels fall sharply. Enzyme induction was tested in mycelium grown on 40 mM-glycerol + 0.2% (w/v) quinic acid pH 6.5, and constitutive enzyme production in mycelium grown on glycerol alone.

This procedure proves a highly sensitive and reliable qualitative test for the presence of enzyme activities, but can be misleading quantitatively. It has been used in an *in vitro* complementation reaction with mycelium of strains known to lack one of each of the three enzymes to identify *qut* mutant enzyme deficiencies, to isolate and analyse constitutive mutants and to test for dominance in heterozygous diploids.

Reversion of qut non-inducible mutants. Spores were plated (approximately 10⁶ per dish) on defined medium with quinic acid at pH 6.5 (1%, w/v) as carbon source, briefly exposed to UV light at a dose permitting 75% survival, and incubated for up to 4 d. Very few colonies appeared for strains plated without irradiation, indicating the stability of the mutant alleles. Revertant strains recovered after irradiation were tested for growth on quinic acid and constitutive enzyme formation was examined by use of the qualitative test following growth on glycerol minimal agar.

RESULTS

Isolation and analysis of further non-inducible qut mutants

A large number of new mutant strains unable to utilize quinic acid as sole carbon source for growth were isolated by filtration-enrichment (Armitt *et al.*, 1976). These mutants represent

lesions in the metabolism of quinic acid to tricarboxylic acid (TCA) cycle intermediates and in the gluconeogenic metabolism of these intermediates for biosynthesis. Mutant strains deficient in gluconeogenesis were identified by their failure to utilize acetate for growth (Armitt *et al.*, 1976). The remaining mutant strains may have lesions in the uptake or metabolism of quinic acid to protocatechuic acid (PCA) or in the further metabolism of PCA to TCA cycle intermediates, and these two classes were separated by the qualitative test for the overall reaction from quinic acid to PCA. This procedure identified 140 specific quinate non-utilization (*qut*) mutants and 168 mutants deficient in the further metabolism of PCA. Some of the latter mutants showed an immediate strong positive reaction when mycelium grown on glycerol and quinate was tested, indicating the accumulation of PCA due to deficiency in PCA oxygenase.

The further characterization of the specific quinic acid non-utilization (*qut*) mutants was based upon a second application of the qualitative test in an *in vitro* enzyme complementation procedure. Samples of quinate-induced mycelium of three mutant strains each known to lack a single enzyme (*qutB42*, dehydrogenase; *qutE208*, catabolic 3-dehydroquinase; *qutC119*, dehydratase), were separately combined with quinate-induced mycelium of each of the new *qut* mutants in the reaction system. A series of reconstruction experiments showed that induced mycelium of the known mutants with different single enzyme deficiencies complemented one another to complete the overall reaction and produce PCA from quinic acid. The combination of mutants with the same enzyme deficiency did not yield significant PCA formation and mycelium of induced mutant strains did not inhibit the production of PCA by induced mycelium of wild-type controls. Interestingly, mycelium of the previously identified non-inducible mutant strains *qutA4* and *qutD8* (Hawkins *et al.*, 1984) yielded no PCA formation in the qualitative test, even after prolonged incubation of the samples either separately or together, and did not prevent PCA production by wild-type induced mycelium. Finally, and as expected, no PCA was detected when mycelium of *qutA4* and *qutD8* grown in the presence of quinic acid was combined with induced mycelium of each of the single enzyme deficient mutant strains described above. Thus the characteristic inability of non-inducible mutants to produce all three of the quinic acid specific enzyme activities can be demonstrated by the *in vitro* enzyme complementation technique.

One half (65) of the new *qut* mutants were non-inducible for all three enzyme activities. Some mutants could not be resolved, either due to a 'leaky' phenotype in the qualitative test or to ambiguous reactions with the known strains, but the loss of a single enzyme activity in the remaining mutants (31 *qutB* and 17 *qutC*) was identified with confidence. Five new *qutE* mutants deficient in catabolic 3-dehydroquinase were found. These mutants, like *qutE208*, were leaky in the qualitative test due to the presence of the isoenzyme biosynthetic 3-dehydroquinase, but gave a strong positive result when combined with *qutB* and *qutC* mutant strains. Since our major interest was the acquisition of further non-inducible mutant strains, the new *qut* mutants with apparent single enzyme deficiencies were not characterized further.

A small number of non-inducible mutants were assayed for the three quinate specific enzyme activities following transfer of glucose-grown mycelium to quinic acid growth medium of pH 6.5. None of the five strains examined (*qut314*, 312, 331, 303 and 365) contained any detectable activity of each of the three enzymes. Two other strains (*qut434*, 435) which were subsequently found non-complementary to *qutB42* in heterozygous diploid strains also lacked dehydrogenase activity.

Analysis of the non-inducible qut mutant strains. Complementation analysis in heterozygous diploid strains was used to characterize the new non-inducible *qut* mutants, and the data confirmed the value of the qualitative test in the classification of mutants by the *in vitro* enzyme complementation procedure. A few of the new *qut* strains (5 in 74) were not fully resolved, and the possibility that these are *qutE* or *qutC* mutants has not been tested. A further 7 strains proved to be *qutB* mutants, and thus the accuracy of the qualitative test in the identification of non-inducible *qut* mutants was over 80% (62 in 74). All of the seven *qutB* and five possible *qutE* or *qutC* mutants were recessive to wild-type.

Amongst 65 non-inducible mutants, 9 contained recessive alleles at the *qutD* locus. These

mutants did not grow on quinic acid media at pH 6.5 but all grew normally on quinic acid when tested at pH 3.5 and produced all three quinic acid specific enzyme activities when induced at this pH. This result strongly suggested that *QUTD* encodes an essential component of a permease required to transport quinate ion into mycelium from media at pH 6.5 (Whittington *et al.*, 1987).

The remaining 56 non-inducible mutants proved of particular interest with respect to their dominance. A minority (including *qutA303*) appeared fully recessive, since when combined with wild-type (*QUT*) and the *qutD8* or *qutB42* mutant strains, the resulting heterozygous diploids exhibited full wild-type growth. The recessive mutants yielded heterozygous diploids with *qutA4* which failed to grow on quinic acid and are therefore identified as *qutA* alleles. The majority of the mutants exhibited partial dominance and growth on quinic acid of heterozygous diploids with wild-type (*QUT*), *qutD8* or *qutB42* was poor compared to wild-type, but stronger than that of homozygous double mutant strains or of the haploid mutant strains. This phenotype is characteristic of the *qutA4* mutant and all of these partially dominant mutants yielded heterozygous diploids with *qutA4* which failed to grow on quinic acid. Thus the majority of the new non-inducible *qut* strains are identified as *qutA* mutants.

A minority of non-inducible *qut* mutants (*qut341*, 365, 373, 382, 405) together with one mutant (*qut214*) isolated previously (Hawkins *et al.*, 1985) appeared fully dominant since heterozygous diploid strains with wild-type (*QUT*), or any other *qut* mutant strain, did not grow on quinic acid medium. The dominance of *qut341* and *qut365* was confirmed by the absence of all three enzyme activities in heterozygous diploid strains (data not shown).

Recombination analysis of the dominant non-inducible qut mutants. The majority of non-inducible mutants isolated exhibit a degree of dominance in heterozygous diploid strains and are functionally allelic to *qutA*, and therefore it seemed likely that the dominant mutations may also be at the *qutA* locus. This was tested by crossing the dominant mutants to a recessive mutant *qutA303* and selecting for prototrophic *QUT* recombinants on quinic acid medium. Three of the dominant non-inducible mutants (*qut214*, 341, 382) were found very tightly linked (RF 0.05% or less) to *qutA303* but three others (*qut365*, 373, 405) recombined at a readily detectable rate (RF 2.5%) and thus are at sites outside the *QUTA* gene locus. Two of these mutants (*qut365* and *qut373*) were mapped 4.6 and 5.0 units respectively from *qutD8* and to the right of *QUTA* (see Fig. 1). Further properties of these dominant non-inducible mutants which strongly suggest their location in *QUTR* are described below.

Reversion of non-inducible qut mutant strains and the isolation and analysis of constitutive mutant strains

The initial interpretation of *QUTA* in *A. nidulans* was a repressor gene equivalent to *QA-1S* in *N. crassa*, and that of *QUTD* an activator gene equivalent to *QA-1F* (Hawkins *et al.*, 1984). Work in the *N. crassa QA* system has shown that a high proportion of prototrophic *QA* revertants of non-inducible *qa-1S* mutant strains selected by growth on quinic acid are constitutive mutants, and that such mutants were not found amongst *qa-1F* revertants (Giles *et al.*, 1985). We therefore attempted to isolate constitutive mutants in *A. nidulans* following the same strategy and reverting *qutA* mutant strains. Altogether 150 revertants of the partial dominant mutant *qutA4*, 250 revertants of the dominant mutant *qut214* and 100 revertants of various recessive mutants (*qutA303*, 324, 361, 444) were screened, and only a single constitutive strain was found. A small number of revertants of *qutD312* were isolated and two constitutive strains recovered amongst 30 examined.

Analysis of the single constitutive revertant obtained in a *qutA* strain (*qutA4* r3) proved complex and will be described in detail elsewhere. The constitutive phenotype is conferred by a non-allele-specific unlinked recessive suppressor of *qutA* mutations. The suppressor is not effective when combined with the dominant mutation *qutA214* and does not confer constitutivity in a wild-type (*QUTA*) strain.

Reversion of qutD mutant strains and the identification of a linked repressor gene, QUTR. Following the unexpected observation that reversion of *qutD312* yielded constitutive strains,

Table 1. Identification of the repressor gene *qutR* in *A. nidulans*

The wild-type strain in the first cross was R21 (*pabaA1 yA*) and in the test crosses *rec6* (*pyroA4; qutR16*) was also crossed to R21 and *rec86* (*yA; pyroA4; qutR16*) was crossed to R10 (*biA1; wA3; galA1*). The pooled data include those for *qutD312* r16 and a series of further crosses between R21 and the constitutive revertant strains *qutD312* r35, *qutD8* r1, *qutD331* r18 and *qutD439* r3.

Cross	Classes of progeny	Phenotype		No. of progeny		Genotype
		Growth on quinic acid	Enzyme formation	r16	Pooled data	
<i>qutD312</i> r16 × wild-type	1. Wild-type	+++	Inducible	68	293	P <i>QUTD QUTR</i>
	2. <i>qutD312</i> r16	(+)	Constitutive	63	251	P <i>qutD312 qutR16</i>
	3. <i>qutD312</i>	—	Non-inducible	2	5	R <i>qutD312 QUTR</i>
	4.	+++	Constitutive	4	10	R <i>QUTD qutR16</i>
				—	—	
				137	559	
<i>Test crosses</i>				<i>rec6</i>	<i>rec86</i>	
Class 4 (<i>rec6; rec86</i>) × wild-type		+++	Inducible	23	40	<i>QUTD QUTR</i>
		+++	Constitutive	27	32	<i>QUTD qutR16</i>
				—	—	
				50	72	

further revertants of a series of *qutD* mutants (*qutD8*, 312, 331, 436, 439) were selected and a high proportion of constitutive strains recovered, 181 in 321 revertants tested. The constitutive strains all had similar phenotypes, growing very poorly on quinic acid, although mycelium grown on glycerol produced a strong positive reaction in the qualitative test, indicating substantial levels of enzymes for the overall reaction from quinic acid to PCA. In marked contrast, strongly growing revertants were not constitutive and growth in the presence of quinic acid was required to induce enzyme activities. These strains probably result from back-mutation at the *qutD* locus.

In order to establish the genetic basis for constitutive enzyme formation, one of the constitutive revertant strains (*qutD312* r16) was crossed with wild-type and the progeny examined for growth on quinic acid and for enzyme formation using the qualitative test (Table 1). The data show that the revertant was a double mutant and the second mutation identified a new gene locus *qutR* which controls induction of the three specific quinic acid enzymes and is closely linked to *qutD*. The constitutive mutation is fully recessive since a heterozygous diploid (*qutR16/QUTR*) showed no trace of overall enzyme activity in the qualitative test when grown in the absence of quinic acid. A further 16 recessive constitutive mutants combined with a *qutR16* produced diploid strains which grew normally on quinic acid and exhibited the constitutive mutant phenotype. Thus, each of the constitutive revertant strains tested contains a new mutation functionally allelic to *qutR16*.

In summary, analysis of the constitutive revertants has shown that the constitutive phenotype results from a recessive mutation in the gene *qutR* which is linked to the *QUT* gene cluster. Since recessive mutations confer a loss of repression the *QUTR* gene is interpreted to encode a repressor protein and is formally designated *QUTR*, quinic acid utilization repressor.

Reversion of the linked dominant non-inducible strains. Prototrophic (*QUT*) revertants of the strain *qut365*, which maps to the *qutR* locus, were selected by plating suspensions of conidiospores on quinic acid medium. The strain reverted spontaneously at a high rate (45 *QUT* colonies from 10⁶ spores) and this rate was increased by exposure to a low dose of UV irradiation (150 colonies from 10⁶ spores). Revertants were screened for enzyme formation in the qualitative test following growth on glycerol and a high proportion (95%) found constitutive. It remains to be established that the mutations conferring constitutivity are at second sites which map close to the original dominant mutation. However, the properties of the dominant mutant in its instability and in yielding a high frequency of constitutive revertants are exactly the same as the repressor *qa-1S⁻* and *qa-1S^c* strain in *N. crassa* (Giles *et al.*, 1985), encouraging the provisional conclusion that they are in the repressor gene locus *QUTR* in *A. nidulans*.

Table 2. *Effect of the constitutive mutation qutR16 upon enzyme formation in A. nidulans*

Mycelium grown in liquid culture for 16 h at 37 °C in 20 mM-glucose was harvested aseptically by filtration and the pad rinsed with warm medium without carbon source. Each pad of mycelium was divided and samples were transferred to fresh warm growth media containing the carbon sources indicated. The cultures were incubated for a further 5 h at 37 °C, then mycelium was harvested, and the pad washed in extraction buffer and stored at -20 °C until cell-free extracts were prepared (see Methods).

Strain	Genotype	Glucose-grown mycelium transferred to*	Relative enzyme specific activity†		
			Dehydrogenase (1)	Dehydroquinase (2)	Dehydratase (3)
Wild-type	<i>QUTD QUTR</i>	Quinic acid	1.00 (0.25)	1.00 (38.8)	1.00 (0.17)
		Glycerol + quinic acid	0.60	0.55	1.00
		Glycerol	0.04	0.02	0
		Glucose + quinic acid	0.16	0.07	0.18
		Glucose	0	0	0
Wild-type	<i>QUTD QUTR</i>	Quinic acid	1.00 (0.27)	1.00 (35.8)	1.00 (0.14)
<i>qutD312</i>	<i>qutD312 QUTR</i>	All conditions	No enzyme activity found		
<i>qutD312 r16</i>	<i>qutD312 qutR16</i>	Quinic acid	0.74	2.42	5.57
		Glycerol + quinic acid	1.19	1.50	2.86
		Glycerol	1.11	2.12	3.36
		Glucose + quinic acid	0.74	0.91	1.43
		Glucose	0.52	0.68	1.00
Wild-type	<i>QUTD QUTR</i>	Quinic acid	1.00 (0.29)	1.00 (46.2)	1.00 (0.27)
rec6(Table 1)	<i>QUTD qutR16</i>	Quinic acid	1.28	0.88	0.74
		Glycerol + quinic acid	1.28	0.85	1.19
		Glycerol	1.31	1.44	2.30
		Glucose + quinic acid	0.72	0.55	0.78
		Glucose	0.55	0.45	0.67

* The concentrations of carbon sources were: quinic acid pH 6.5, 0.5% (w/v); glycerol, 40 mM, or glucose, 20 mM, alone or with quinic acid pH 6.5 at 0.2% (w/v).

† Relative enzyme specific activities are shown as the fraction of each of the specific activities in a quinate-grown wild-type control strain R153 (*wA3*; *pyroA4*). Each block of results represents a particular set of experiments which included the induced wild-type control strain. Enzyme specific activities (for the absolute values shown in parentheses for the wild-type) are expressed as follows: (1) dehydrogenase, $\mu\text{mol product min}^{-1} (\text{mg protein})^{-1}$; (2) catabolic dehydroquinase (extract heated at 70 °C for 10 min to inactivate the biosynthetic isoenzyme), units mg^{-1} (1 unit is a ΔA_{240} of 0.1 per min at 37 °C); (3) dehydratase, $\mu\text{mol product min}^{-1} \text{mg}^{-1}$.

Control of quinic acid specific enzyme formation

Catabolite repression. Induction of the quinic acid specific enzymes in wild-type is diminished when alternative carbon sources are present in growth medium with quinic acid (Hawkins *et al.*, 1984). The possibility that compounds such as glucose decrease induction by preventing the uptake of quinic acid into mycelium was tested by growth of mycelium of a permease mutant *qutD8* on glucose followed by induction on quinic acid at pH 3.5. In this situation no permease function is required for the uptake of quinic acid and the quinate dehydrogenase was 50% repressed and the catabolic 3-dehydroquinase and dehydratase were fully repressed (data not shown). It was also found that enzyme formation was partially repressed by glucose in *qutR* constitutive strains (Table 2). These observations confirm that enzyme induction by quinic acid is subject to a second level of control by carbon catabolite repression when alternative carbon sources such as glucose are present in the growth medium. They strongly suggest that catabolite repression is not exerted in the uptake of quinic acid but occurs within the mycelium where it is presumably mediated by the concentration of cAMP (Pall, 1981).

Constitutive expression of the quinic acid specific enzymes in *qutR16* strains. The mutation *qutR16* conferred constitutive expression of high levels of each of the three specific quinic acid enzyme activities when strains were grown in the absence of quinic acid (Table 2). Growth in the presence of quinic acid did not greatly increase enzyme levels compared to those on glycerol

Fig. 1. The map of the *qut* gene cluster is from data in Table 3. The cluster was mapped in chromosome VIII in a cross between *qutD312 qutR16* and a strain containing *galG2 fwA1 acuC307* from which 500 progeny were analysed (data not shown). A recombinant *qutD qutR galG fwA* was crossed with an *ornB* strain and 300 progeny were scored (data not shown). Selection of *ORNB QUTD* recombinants from the second cross enables deduction of the gene order:

	+	<i>qutD</i>	<i>qutR</i>	<i>galG</i>	<i>fwA</i>
<i>ornB</i>	+	+	+	+	+

The distribution of the non-selected markers in 212 recombinants taken at random and a further 44 *fwA* recombinants was:

<i>qutR</i>	<i>galG</i>	<i>fwA</i>	All recombinants	<i>fwA</i> recombinants
-	-	-	6	3
-	-	+	0	-
-	+	-	0	1
-	+	+	0	-
+	-	-	23	26
+	-	+	5	-
+	+	-	7	14
+	+	+	171	-
			212	44

Thus the majority of recombinants were non-constitutive (*QUTR*) and all of the constitutive (*qutR*) recombinants were also *galG* and *fwA*. The predicted proportion of constitutive recombinants is 4.4 in 100, of which the majority are expected *fwA*, and amongst *fwA* recombinants 4 in 25 are expected constitutive. If the *QUTR* locus were between *ORNB* and *QUTD* the predicted proportion of constitutive recombinants would be 4 in 12.

alone, indicating that the mutation fully derepresses quinic acid mediated gene expression. The presence of high enzyme levels in the revertant strain *qutD312 qutR16* contrasts strongly with the very poor growth produced by the strain on quinic acid, and argues that growth is limited by the defect in quinic acid uptake resulting from the *qutD* mutation. Failure of *qutD* mutants to induce enzyme activities can therefore be understood since quinic acid does not enter the mycelium at a sufficient rate to become an effective inducer (Whittington *et al.*, 1987).

Fine-structure mapping of the *QUT* gene cluster

Previous work has established that the *QUT* genes are tightly linked in a cluster on chromosome VIII in *A. nidulans* (Hawkins *et al.*, 1982). The isolation of the constitutive mutation *qutR16* in the double mutant strain *qutD312 qutR16* therefore presented an opportunity to map the genes in the cluster by three-point crosses using the *qutR16* mutation as a flanking marker. The distribution of the constitutive marker *qutR16* in the *QUT* recombinants orders the *QUT* genes with respect to *qutD* and *qutR*. The data map *qutC* distal to *qutD* with

Table 3. *Fine-structure mapping of the QUT gene cluster in A. nidulans*

The double mutant strain *qutD312 qutR16* was crossed with each of the *qut* single mutants and suspensions of ascospores from single hybrid cleistothecia were plated on glucose medium to estimate the viable count and on quinic acid medium to select prototrophic *QUT* recombinants. The distribution of the *qutR* marker in a random sample of the *QUT* recombinants was determined using the qualitative test on glycerol-grown mycelium. In each cross two or more cleistothecia were analysed and certain crosses were repeated with different alleles at the third *qut* gene locus. The numbers of *QUT* recombinants were multiplied by two, on the argument of symmetry of reciprocal classes in inter-genic crosses, to provide an estimate of the RF between *QUTD* and each of the *QUT* genes.

Third <i>qut</i> gene in the cross	10 ⁻³ × Total progeny tested	Total recombinant progeny	RF (%)	Phenotype of <i>QUT</i> recombinants			Gene order
				Inducible <i>QUTR</i>	Constitutive <i>qutR</i> ⁻	Total tested	
<i>qutA303</i> 1	36.0	1400	3.89	1	49	50	
	2	53.6	1544	2.88	51	52	
<i>qutA4</i> 1	12.4	448	3.61	0	50	50	
	2	43.6	624	1.43	52	52	
	145.6	4016	2.76	2	202	204	<i>D-A-R</i>
<i>qutB42</i> 1	84.8	304	0.36	11	26	37	
	2	37.7	324	0.86	26	29	
	3	142.0	504	0.35	25	32	
	264.5	1132	0.43	21	77	98	<i>D-B-R</i>
<i>qutC113</i> 1	67.2	808	1.20	55	2	57	
	2	32.5	448	1.38	13	54	
<i>qutC119</i> 1	205.8	416	0.20	50	4	54	
	2	62.9	1210	1.92	5	48	
	368.4	2882	0.78	189	24	213	<i>C-D-R</i>
<i>qutE208</i> 1	54.2	544	1.00	0	50	50	
	2	126.8	1520	1.20	48	50	
	181.0	2064	1.14	2	98	100	<i>D-E-R</i>

respect to *qutR*, and place *qutA*, *qutB* and *qutE* in the interval between the two markers. The RF values unambiguously order the genes in the sequence *qutD*, *qutB*, *qutE*, *qutA* and *qutR* (Table 3, Fig. 1).

The striking result is the location of *QUTC*. Previous work had indicated the location of *QUTC* between *QUTB* and *QUTE* on the basis of the hybridization of defined *N. crassa* *QA* gene probes to *A. nidulans* DNA cloned in recombinant phage λ -Q1 (Hawkins *et al.*, 1985). This result is not consistent with the genetic data described above and DNA sequences in λ -Q1 or any derived sub-clone do not transform *qutC* mutants, although *qutE*, *qutD*, *qutB* and *qutA* mutants are transformed to wild-type (Francisco Da Silva *et al.*, 1986; Whittington *et al.*, 1987; Beri *et al.*, 1987; A. R. Hawkins and others, unpublished). Taken together these data are consistent with the physical presence and biological integrity of the *A. nidulans* genes *QUTD*, *QUTB*, *QUTE* and *QUTA* in the λ -Q1 clone and in an order which correlated precisely with that derived by genetic mapping.

Mapping the qut gene cluster in linkage group VIII. Crosses between the double mutant strain *qutD312 qutR16* and strains with a series of markers in chromosome VIII accurately map the cluster within the chromosome (Fig. 1). The orientation of the cluster on chromosome VIII was determined by selection of *ORNB QUTD* recombinants from a cross between an *ornB7* strain and a strain *qutD312 qutR16 galG2 fwA1* and analysis of the distribution of the *qutR*, *galG* and *fwA* markers (Fig. 1).

Table 4. Enzyme formation in heterozygous diploid strains

A series of recombinant strains, denoted *qutA4-1* to -8 and *qutB42-1* to -8, were isolated in the genotypic backgrounds R153 *wA3; pyroA4* or R21 *pabaA1 yA* from crosses between the original mutant strains R21 *qutA4* or R21 *qutB42* and R153. Diploid strains heterozygous for *qutA4* and *qutB42* were constructed between these recombinants as shown in the table. Each of the diploid strains was grown in liquid culture with glucose as a carbon source following the protocol described in Table 2 and transferred to fresh growth medium with 0.1% (w/v) quinic acid at pH 6.5 and incubated for a further 5 h. Mycelium was harvested, cell-free extracts were prepared and enzyme was assayed as described in Table 2 except that the extracts were dialysed against 10 mM-potassium phosphate buffer pH 7.2, 1 mM-EDTA for 3 h before assay. Each block of results refers to a group of diploid strains treated strictly in parallel together with different cultures of the same wild-type diploid strain (R153/R21). Units of specific enzyme activity are defined in Table 2.

Heterozygous diploid strains			Enzyme specific activities		
Serial no.	Genotype		Dehydrogenase (1)	Dehydroquinase (2)	Dehydratase (3)
	R153	R21			
WT (1)	<i>QUT</i>	<i>QUT</i>	0.47	84.4	0.43
1	<i>qutB42-1</i>	<i>qutA4-8</i>	0.26	91.2	0.45
2	<i>qutB42-1</i>	<i>qutA4-7</i>	0.21	77.6	0.35
3	<i>qutB42-1</i>	<i>qutA4-6</i>	0.23	93.8	0.42
WT (2)	<i>QUT</i>	<i>QUT</i>	0.31	112.0	0.27
5	<i>qutB42-2</i>	<i>qutA4-5</i>	0.18	117.0	0.40
6	<i>qutB42-3</i>	<i>qutA4-5</i>	0.13	78.3	0.27
7	<i>qutB42-4</i>	<i>qutA4-5</i>	0.15	97.1	0.27
8	<i>qutA4-3</i>	<i>qutB42-5</i>	0.20	100.0	0.33
WT (3)	<i>QUT</i>	<i>QUT</i>	0.53	77.1	0.50
9	<i>qutB42-2</i>	<i>qutB42-5</i>	0.20	58.8	0.47
10	<i>qutA4-1</i>	<i>qutB42-5</i>	0.19	54.4	0.35
11	<i>qutA4-1</i>	<i>qutA42-6</i>	0.24	62.5	0.42
12	<i>qutA4-1</i>	<i>qutB42-7</i>	0.23	71.0	0.50
WT (4)	<i>QUT</i>	<i>QUT</i>	0.58	97.0	0.20
4	<i>qutB4-21</i>	<i>qutA4-5</i>	0.16	78.0	0.17
13	<i>qutA4-1</i>	<i>qutB42-8</i>	0.21	64.0	0.18

Enzyme formation in heterozygous diploid strains

In wishing to study potential gene interactions in heterozygous diploid strains we thought it essential to establish that the enzyme levels induced by quinic acid reflect the specific *QUT* genotype of strains investigated and are not subject to variation caused by unidentified modifying factors in the genetic background. In order to test this hypothesis, enzyme levels were measured in a series of heterozygous diploid strains constructed between different haploid recombinant strains isolated from sexual crosses and carrying the *qutA4* or *qutB42* mutant alleles in the complementary genotypes (Table 4). The data were subjected to an unbalanced analysis of variance using the methods of Baker & Nelder (1978). The statistical analysis (not shown) indicated that the activity of quinate dehydrogenase in heterozygous diploids (*qutB42/QUTB*) has a highly significant difference from that in the homozygous diploid (*QUTB/QUTB*) as is expected for a mutant strain, and that the reduced levels do not differ if the *qutB42* mutant allele is in the R21 or R153 genetic backgrounds. Moreover, when the non-inducible catabolic *qutA4* mutation is in either the R21 or the R153 background the activities of catabolic 3-dehydroquinase (2) and dehydratase (3) in heterozygous diploids (*qutA4/QUTA*) do not differ significantly from the homozygous diploid (*QUTA/QUTA*) control. Overall, the analysis shows that there is no major contribution to variance from potential modifying genes segregating in the genetic background and that the phenotypes observed can be directly attributed to the *QUT* genotypes.

DISCUSSION

This paper identifies genes regulating expression of the quinic acid utilization (*QUT*) gene cluster in *Aspergillus nidulans* and extends the model previously proposed in which two genes regulate expression of the enzyme structural genes (Hawkins *et al.*, 1984). Recessive non-inducible mutants at the *QUTA* locus identify the gene as normally having a positive role, activating the expression of the structural genes. Recessive constitutive mutants have identified a new gene locus *QUTR* distinct from *QUTA* and the other *QUT* gene loci, but closely linked ($RF = 4.4\%$) to the *QUT* gene cluster. Thus the positively acting *QUTA* gene encodes an activator protein and the negatively acting *QUTR* gene a repressor protein. Several hypotheses can be suggested for the precise mechanism by which these two proteins may interact with each other, with the inducer and at the promoter regions of the structural genes. Rapid progress in the molecular analysis of the system will enable refinement of the model (A. R. Hawkins and others, unpublished).

The formal map of the *QUT* gene cluster in *A. nidulans* (Fig. 1) is consistent with the molecular analysis of the cloned genes (A. R. Hawkins and others, unpublished). Comparison of the genomic organization of the *QUT* cluster in *A. nidulans* with the corresponding *QA* gene cluster in *Neurospora crassa* (Giles *et al.*, 1985) is therefore of interest. Both systems include five quinate inducible genes and an activator and a repressor regulatory gene. Two of the genes in *N. crassa*, *QA-X* and *QA-Y*, have no identified function. We find that the *QUTD* permease gene in *A. nidulans* shows sequence homology to *QA-Y* in *N. crassa* (R. Geever, personal communication), supporting the deduction that *QA-Y* encodes a permease (Giles *et al.*, 1985). Major differences are found between the two systems since in *A. nidulans* (Fig. 1) the *QUTC* gene (dehydratase = *QA-4*) and the repressor gene *QUTR* (= *QA-1S*) are displaced and map at significant distances from the other tightly linked genes. Separation of the two regulatory genes requires that each is under the control of an independent promoter whereas in *N. crassa* the two corresponding genes are transcribed from a common promoter region. It remains to be discovered if this difference in the location of the regulatory genes in *A. nidulans* may be correlated with differential physiological controls acting at each of the promoters. Previous work has demonstrated transcription control of the catabolic 3-dehydroquinase *QUTE* gene (Francisco Da Silva *et al.*, 1986) and it is likely that the other structural genes are similarly controlled. The nature of the control of the two regulatory genes is currently under investigation.

Three different classes of non-inducible activator *qutA* gene mutation have been found in *A. nidulans* based upon growth of heterozygous diploid strains on quinic acid media. In one, the diploids grow normally, enzyme activities are fully induced and the mutants are thus recessive. In a second, the diploids do not grow, enzymes are not induced and the mutants are thus fully dominant. In contrast, the majority of mutants appear partially dominant in that growth of the diploids on quinic acid is decreased. However, although induced enzyme activities appear similarly decreased in heterozygotes (Hawkins *et al.*, 1984), statistical analysis shows that the observed values for 3-dehydroquinase and dehydratase fall within the range of variation for wild-type diploid controls (Table 4). Therefore this major class of non-inducible mutants are recessive to the wild-type (*QUTA*) with respect to enzyme induction for 3-dehydroquinase and dehydratase although incompletely recessive in growth of heterozygous diploids. It is possible that the reduced growth reflects a differential effect of the *qutA4* mutant gene product upon the expression of *QUTB* and/or *QUTD*, and we are currently investigating this possibility. These observations are like those in *N. crassa* where the non-inducible *qa-1F* mutants are all classified as recessive in heterokaryon tests. However, such tests are acknowledged as unsatisfactory because the precise allele ratio is unknown in the heterokaryons (Giles *et al.*, 1985).

The repressor gene *QUTR* was discovered as second-site mutations in poorly growing revertants of permease deficient (*qutD*) strains. The constitutive mutations in *qutR* are fully recessive and most probably represent loss of repressor activity. The small number of non-inducible dominant mutants which map consistent with their location in *qutR* revert at a high frequency to produce constitutive mutants. The dominance and location of these presumed second-site *qutR* mutations remain to be established, but exactly parallel the reversion of dominant mutations in the repressor gene *qa-1S* in *N. crassa* (Giles *et al.*, 1985). We are actively

pursuing the isolation of the *QUTR* repressor gene. The single constitutive strain recovered among revertants of *qutA* mutants proved due to a novel unlinked mutation, and the apparent absence of constitutive mutations at the *QUTA* gene locus, also found for the corresponding locus in *N. crassa* (Giles *et al.*, 1985), remains a tantalizing puzzle. Among several interpretations of this observation we note the possibility that the *QUTA* gene product is unable to induce enzyme formation when acting alone and requires a particular conformational state of the *QUTR* gene product as a co-activator.

The similarities and differences between the *QUT* gene cluster in *A. nidulans* and the *QA* cluster in *N. crassa* are fascinating. The opportunity is close at hand for a detailed comparison of the molecular organization of the cluster (A. R. Hawkins and others, unpublished) and of the *QUTA* activator protein (Beri *et al.*, 1987) in *A. nidulans* with the cluster and *QA-1F* activator protein in *N. crassa* (Giles *et al.*, 1985 and personal communication).

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