Selective overexpression of the *QUTE* gene encoding catabolic 3-dehydroquinase in multicopy transformants of *Aspergillus nidulans*

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The three enzymes necessary to catabolize quinate to protocatechuate are inducible by quinic acid, and transcription of their corresponding genes is controlled by the action of a positively acting activator gene and a negatively acting repressor gene. Transformed strains of *Aspergillus nidulans* containing multiple copies of the activator gene (*QUTA*) but single copies of the other *QUT* genes retain normal regulation of the gene cluster and do not show any overexpression of the three quinic acid catabolic enzymes. Transformed strains containing equal multiple copies of the activator gene (*QUTA*) and *QUTE* (encoding catabolic 3-dehydroquinase), but single copies of the other *QUT* genes, retain normal regulation of the *QUT* gene cluster, but selectively overexpress the *QUTE* gene upon quinic acid induction. Data are presented that strongly suggested that the gene *QUTG*, which is physically located within the *QUT* gene cluster and for which no function has been identified, is not required for expression of the gene cluster and does not encode a chlorogenic acid esterase.

INTRODUCTION

Aspergillus nidulans grows strongly on quinic acid as sole carbon source, and six genetically characterized genes required to catabolize quinate to protocatechuate are linked in a tight cluster on chromosome VIII (Hawkins *et al.*, 1982, 1984, 1985; Grant *et al.*, 1988). These genes encode quinate dehydrogenase (*QUTB*), 3-dehydroquinase (*QUTE*) and dehydratase (*QUTC*) (Hawkins *et al.*, 1982), a quinate permease, *QUTD* (Whittington *et al.*, 1987), a positively acting regulatory protein (*QUTA*; Beri *et al.*, 1987) and a protein with a negative regulatory role (*QUTR*; Grant *et al.*, 1988). Two of the metabolic intermediates in the catabolism of quinate, 3-dehydroquininate and dehydroshikimate, are also produced in the common pathway for the synthesis of the aromatic amino acids (Kingham & Hawkins, 1982). The enzymes acting on these substrates in the aromatic amino acid biosynthetic pathway are specified by a pentafunctional polypeptide encoded by the *AROM* locus, which itself shows a complex evolutionary relationship with the *QUT* gene cluster and the equivalent monofunctional bacterial *aro* loci (Charles *et al.*, 1985, 1986; Hawkins, 1987; Hawkins *et al.*, 1988).

In order to understand the genetic and molecular mechanisms which regulate and co-ordinate the expression of the potentially competing *QUT* and *AROM* pathways, quinate-non-utilizing mutants have been analysed. The model for induction by quinate arising from this analysis proposes specific binding of the *QUTA* protein to defined sequences which have been identified in 5'-non-translational regions of the *QUT* structural genes (Grant *et al.*, 1988; Hawkins *et al.*, 1988).

A recombinant λ -phage (λ Q1) containing the *QUTA*, *QUTB*, *QUTD* and *QUTE* genes and a genetically uncharacterized seventh gene, *QUTG* (which has a quinate-inducible message but no known function), has been isolated and physically characterized (Hawkins *et al.*, 1985, 1988; Da Silva *et al.*, 1986; Beri *et al.*, 1987; Whittington *et al.*, 1987). We have previously used subclones of λ Q1 to make transformants of *A. nidulans* which have increased copy numbers of the *QUTE* gene (encoding 3-dehydroquinase) with a single copy of the activator (*QUTA*) and repressor genes (*QUTR*). Such multicopy transformants show normal regulation of the *QUT* gene cluster with no elevation of 3-dehydroquinase upon quinic acid induction, strongly suggesting that the *QUTA* gene product is limiting in induction. We have continued the study of transformed strains containing multiple copies of the *QUTA* (activator) and *QUTE* (3-dehydroquinase) genes, and show that increasing the copy number of the *QUTA* gene, and thereby presumably increasing the concentration of the activator protein, is insufficient to overexpress the *QUT* gene cluster. Using a transformant containing seven copies of *QUTA* and *QUTE* we show that the *QUT* cluster is regulated normally, but that the *QUTE* gene is selectively overexpressed upon quinic acid induction, and that 3-dehydroquinase then constitutes a significant proportion of total cell protein.

MATERIALS AND METHODS

Organisms and materials

The origins of the λ -phage, *A. nidulans* and *Escherichia coli* strains used have been detailed previously (Hawkins

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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et al., 1984, 1985). *A. nidulans* strains R153 *qutA303* (wA3: *pyroA4*: *qutA303*) and G191 (Grant *et al.*, 1988) were crossed; strain 269 (*fwA1*, *pabaA1*, *pyrG189*, *qutA303*) was isolated and used as the recipient in all subsequent transformation experiments with plasmids pAL7ΔG and pAL7ΔGE. Media for the propagation of λ-phage, *A. nidulans* or *E. coli* were those previously reported (Willets *et al.*, 1969; Armitt *et al.*, 1976; Blattner *et al.*, 1977; Hawkins *et al.*, 1984) and materials were obtained as described in Hawkins *et al.* (1984) and Charles *et al.* (1985).

Methods

Recombinant DNA techniques were as previously described (Maniatis *et al.*, 1982) using the manufacturer's recommended conditions for use of enzymes. Southern-blot analysis used the method of Jeffreys *et al.* (1980). *A. nidulans* DNA was prepared by the method of Clements & Roberts (1985). *E. coli* and *A. nidulans* transformations used the methods of Kushner (1978) and Ballance & Turner (1985). SDS/polyacrylamide electrophoresis (SDS/PAGE) was performed by the method of Laemmli (1970).

A 7 kb fragment of DNA produced by a partial restriction-endonuclease-*Bam*H1 digest of λQ1 was isolated, subcloned into *Bam*H1-digested plasmid pBR325 and designated 'pAL7' (Whittington *et al.*, 1987). The 7 kb *Bam*H1 partial digest restriction fragment contains the *QUTA*, *QUTE* and *QUTG* genes, shown as the black, cross-hatched and stippled boxes respectively in Fig. 1. The *QUTG* gene was removed by digesting pAL7 DNA with *Xba*1, followed by self-ligation, yielding plasmid pAL7ΔG. The *QUTE* gene was inactivated by digesting pAL7ΔG DNA with *Xba*1, treating with *Bal*31

and self-ligation after filling in with the Klenow polymerase, yielding plasmid pAL7ΔGE. Fig. 1 summarizes the construction of these plasmids. All plasmids were recovered from ligation mixtures by transformation of *E. coli* strain SK3430, and their correct construction confirmed by digestion with appropriate restriction enzymes.

U.v. mutagenesis of conidiospores and subsequent filter enrichment in minimal medium containing 5 mM-chlorogenic acid as carbon source was as previously described (Armitt *et al.*, 1976).

The copy number of genes transformed into *A. nidulans* was determined by taking 1 μg of denatured chromosomal DNA from wild-type and transformed strains and serially diluting to a final dilution of 1:16. Samples (5 μl) were spotted in triplicate on to a gridded nitrocellulose filter, baked to bind the DNA and probed with α-³²P-labelled 1.7 kb internal *Eco*RI fragment of the *QUTA* gene. Autoradiographs developed after exposure to the nitrocellulose filter were analysed on a Joyce-Loebl Chromoscan 3 scanning densitometer and the data were processed by using the manufacturer's computer software.

RESULTS AND DISCUSSION

Transformation of *qutA* strains

The constructions of plasmids pAL7, pAL7ΔG, and pAL7ΔGE (Fig. 1) were described above. The functional integrity of the *QUTE* gene in plasmids pAL7 and pAL7ΔG was verified by transformation of strain WA53 (*pyrG189*; *pyroA4*; *qutE208*), which lacks 3-dehydroquinase and selection on minimal medium with quinate as sole carbon source (results not shown).

Fig. 1. Construction of plasmids pAL7ΔG and pAL7ΔGE

A 20 μg portion of plasmid pAL7ΔG DNA was linearized with endonuclease *Xba*1 and digested with 10 units of *Bal*31 after phenol extraction, ethanol precipitation and resuspension in appropriate buffers as detailed by Maniatis *et al.* (1982). Samples of *Bal*31-digested plasmid were taken at 10 min intervals over 1 h, the reaction stopped with an excess of 0.2 M-EGTA and, after phenol extraction, ethanol precipitation and resuspension in appropriate buffers, the ends of the linear molecules made flush with 1 unit of Klenow polymerase. After ligation overnight at 26 °C, individual molecules were rescued from ligation mixtures by transforming *E. coli* strain SK3430 and plating on ampicillin plates. A total of 12 individual plasmids were purified and characterized by digestion with *Bam*H1 and *Eco*RI to monitor the extent of the *Bal*31 deletion; one plasmid with a deletion of approx. 1 kb inactivating the *QUTE* gene was designated 'pAL7ΔGE' and used in all subsequent *A. nidulans* transformations. The black, cross-hatched and stippled boxes respectively denote the positions and sizes of the *QUTA*, *QUTE* and *QUTG* genes, and the arrows over the boxes denote the direction of transcription. The symbols B, Bg, E and X denote the positions of restriction-endonuclease recognition sites for *Bam*H1, *Bgl*II, *Eco*RI and *Xba*1 respectively.

Table 1. Enzyme levels in selected strains transformed with plasmid pAL7

Concentrations of carbon sources were as follows: glucose or glycerol, 40 mM; quinic acid, pH 6.5, 0.5% (w/v). †Specific enzyme activities are expressed as follows: (1) dehydrogenase, μmol of product $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$; (2) catabolic dehydroquinase, units $\cdot \text{mg}$ of protein $^{-1}$ (1 unit is a change in A_{240} of 0.1/min at 37 °C); (3) dehydratase, μmol of product $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. The values shown in parentheses after the 3-dehydroquinase values are the relative values compared with the wild-type (R153) induced value, which was taken as 1. The recipient strain for transformation was R153 *qutA303*.

Part	Strain	Carbon source	Enzyme specific activity		
			Quinate dehydrogenase	3-Dehydroquinase	Dehydratase
(a)	R153	Quinic acid	0.14	13 (1)	0.13
	pAL7 A1	Quinic acid	0.12	265 (20.4)	0.06
	pAL7 A2	Quinic acid	0.11	58 (4.5)	0.086
	pAL7 A3	Quinic acid	0.11	63 (4.8)	0.1
	pAL7 A7	Quinic acid	0.11	293 (22.5)	0.03
(b)	R153	Glucose	0	0.9	0
		Glycerol	0.01	1.3	0
		Quinic acid	0.17	22.4 (1)	0.07
	pAL7 A3	Glucose	0.1	1.1	0
		Glycerol	0.1	1.3	0
		Quinic acid	0.17	101 (4.5)	0.13
	pAL7 A7	Glucose	0	4.7	0
		Glycerol	0	2.8	0
		Quinic acid	0.17	547 (24)	0.07

Table 2. Enzyme levels in selected strains transformed with plasmid pAL7ΔG

For definitions and carbon-source concentrations, see the legend to Table 1. The recipient strain for transformation was 269 (*pyrG189*; *qutA303*).

(a)

Strain		Specific activity of 3-dehydroquinase in mycelium grown with:	
		Quinic acid	Glycerol
	R153	49 (1)	0.19
pAL7ΔG	A1	305 (6.2)	0.23
pAL7ΔG	A8	257 (5.2)	0.15
pAL7ΔG	A12	310 (6.3)	0.26

(b)

Strain		Enzyme specific activity		
		Quinate dehydro- genase	3-Dehydro- quinase	Dehydratase
R153	Quinic acid	0.16	37 (1)	0.075
pAL7ΔG-1	Glucose	0.003	1.3	0
	Glycerol	0.007	1.5	0
	Quinic acid	0.17	176 (4.8)	0.063

Strains R153 *qutA303* and 269 were used as recipients in transformation experiments with plasmids pAL7, pAL7ΔG and pALΔGE, and in each case transformants were selected by growth on minimal medium with quinic acid as sole carbon source. Strain R153 *qutA303* had previously been transformed with plasmid pAL7 as part of an exercise to identify and characterize the *QUTA* gene (Beri *et al.*, 1987). Strain 269 was independently checked for its ability to undergo transformation by using the plasmid pDJBIII (Ballance & Turner, 1985), which contains the *Neurospora crassa pyr-4* gene, to complement the *pyrG* mutation and restore growth on minimal medium lacking uracil (results not shown).

Strain 269 was transformed with plasmids pAL7ΔG and pAL7ΔGE and transformants were selected by growth on quinic acid. In each experiment chromosomal DNA was prepared from twelve independently isolated transformants of each strain, and 0.5 µg of this DNA screened in a dot-blot with wild-type R153 control DNA using α-³²P-labelled 1.7 kb *EcoRI* internal fragment from the *QUTA* gene as a probe (results not shown). DNA from pAL7 transformants, showing hybridization signals indicating wild-type single-copy equivalent, and from a range of putative multiple-copy inserts, was subjected to Southern-blot analysis after digestion with restriction endonuclease *Bgl*II and hybridized with the same *QUTA* gene probe. The copy number in two pAL7 transformed strains (pAL7A3 and pAL7A7), was determined by dot-blot analysis as described in the Materials and methods section and found to be 2 for pAL7A3 and 7 for transformant pAL7A7.

Enzyme levels in transformants with multiple copies of *QUTE* and *QUTA* and *QUTG*

Four representative pAL7 transformants (pAL7A1, pAL7A2, pAL7A3 and pAL7A7) were grown with quinic acid as carbon source with wild-type strain R153 as a

control, and the levels of the three *QUT* enzymes were measured in cell-free extracts. Table 1(a) shows that the levels of the quinate dehydrogenase and dehydratase in the transformants do not exceed the levels in the wild-type control, whereas the levels of 3-dehydroquinase in the transformants exceed that of the wild-type control in a manner that is related to copy number: pAL7A3 (copy number 2) is elevated 4.8-fold and pAL7A7 (copy number 7) is elevated 22.5-fold.

The enzyme levels in pAL7A3 and pAL7A7 were measured in cell-free extracts of cultures grown with glucose, glycerol or quinic acid as carbon source with an R153 control, to see whether the elevated levels of 3-dehydroquinase were being regulated normally in response to the presence of quinic acid. The data are summarized in Table 1(b); inspection of this shows that the 3-dehydroquinase enzyme is inducible by quinate and that the relative levels of enzyme present in glucose- or glycerol-grown cultures are not greater than the levels found in the wild-type quinate-induced control. This strongly implies that the *QUTE* genes in the transformants are subject to the same control by *QUTA* protein in response to quinic acid as the wild-type.

Enzyme levels in transformants containing multiple copies of *QUTA* and *QUTE*

In order to discover if multiple copies of the *QUTG* gene are required for the overexpression of the *QUTE* gene in multicopy transformants, the 3-dehydroquinase enzyme levels in three pAL7ΔG transformants were assayed. Transformants pAL7ΔG 1, 8 and 12 were selected on the basis of a dot-blot hybridization signal showing approximately the same intensity as transformant pAL7A7, which contains seven copies of the *QUTA* gene. The transformants were grown with glycerol or quinic acid as carbon source with a wild-type R153 control. Table 2(a) shows the 3-dehydroquinase levels

Table 3. Enzyme levels in selected strains transformed with plasmid pAL7ΔGE

For definitions and carbon source concentrations see the legend to Table 1. The recipient strain for transformation was 269 (*pyrG189; qutA303*).

(a)

Strain	Specific activity of 3-dehydroquinase in mycelium grown with:	
	Quinic acid	Glycerol
R153	49 (1)	0.19
pAL7 GE-A4	55 (1.1)	0.03
pAL7 GE-A10	59 (1.2)	8.00

(b)

Strain	Carbon source	Enzyme specific activity		
		Quinate dehydro- genase	3-Dehydro- quinase	Dehydratase
R153	Quinic acid	0.16	37	0.075
pAL7ΔGE-A4	Glucose	0.005	1.3	0
	Glycerol	0.007	1.2	0
	Quinic acid	0.19	42	0.15

Table 4. Purification of 3-dehydroquinase enzyme from transformant pAL7 A7

Mycelium (R153, 1.25 g; pAL7A7, 23 g) was harvested from minimal medium (with quinate as carbon source) by filtration, washed with deionized water, pressed dry between absorbent paper and stored at -18°C until needed. Frozen mycelium was ground to a fine powder in a mortar and pestle under liquid N_2 , and cell-free extracts were prepared as previously described (Hawkins *et al.*, 1982).

Strain	Step	Total enzyme activity (units)	Volume (ml)	[Protein] (mg/ml)	Specific activity	Yield (%)
R153 control	Crude supernatant	1250	25	0.62	0.1×10^3	100
pAL7A7	Crude supernatant	338250	550	0.53	1.16×10^3	100
pAL7A7	After heat shock	330000	550	0.29	2.07×10^3	97.6
pAL7A7	$(\text{NH}_4)_2\text{SO}_4$ precipitation	300000	20	1.4	10.7×10^3	88.7
pAL7A7	After DEAE-cellulose DE52 chromatography	227200	40	0.045	126×10^3	67.2

Fig. 2. Purification of 3-dehydroquinase from transformant pAL7A7

The molecular-mass standards used were: phosphorylase *b*, 91 kDa; bovine serum albumin 67 kDa; alcohol dehydrogenase, 41 kDa; chymotrypsinogen, 25.1 kDa; cytochrome *c*, 12.38 kDa. The acrylamide concentration of the gel was 12.5% and the sample wells contained: 1 and 2, molecular-mass standards; 3, crude supernatant from a wild-type (R153) extract; 4, crude supernatant from transformant pAL7A7; 5, supernatant post heat shock from transformant pAL7A7; 6, active 3-dehydroquinase fractions eluted from the DEAE-cellulose DE52 column from transformant pAL7A7; 7 and 8, molecular-mass standards. Lanes 3–6 inclusive contained approx. $3 \mu\text{g}$ of total protein.

measured in cell-free extracts and Table 2(b) shows the levels of all three *QUT* enzymes in transformant pAL7AG1 determined in a separate experiment to verify that the enzymes are induced by quinic acid in the same manner as the wild-type. Inspection of Table 2 shows that the *QUTG* gene is not required for selective overexpression of the *QUTE* gene.

We thought it possible that *QUTG* may represent a chlorogenic acid esterase, as chlorogenic acid is a naturally occurring ester of quinic and caffeic acids. Mutants in this putative esterase would be expected to fail to grow with chlorogenic acid as carbon source, but grow normally with quinic acid as carbon source. Growth tests

showed that *A. nidulans* can utilize chlorogenic acid at 5 mM as a carbon source and, after screening 3000 colonies derived from u.v.-treated conidiospores surviving filter enrichment (with chlorogenic acid as carbon source), one mutant unable to utilize chlorogenic acid as carbon source was isolated. This mutant designated, *cut6* (chlorogenic acid utilization), had the expected ability to utilize quinic acid as carbon source. The *cut6* mutation was recessive, showed independent assortment in crosses with *qut* mutants, and showed linkage with the yellow marker in haploidization analysis, locating the *cut6* mutation on chromosome I. Since the quinic acid-utilization gene cluster maps on chromosome VIII, the *cut6* mutation cannot be an allele of the *QUTG* gene, suggesting that *QUTG* does not code for a chlorogenic acid esterase.

Enzyme levels in transformants containing multiple copies of *QUTA*

In order to determine whether strains transformed with plasmid pAL7ΔGE (containing *QUTA* only) are capable of overexpressing the *QUT* genes, two transformants showing dot-blot hybridization signals equivalent to the signal from transformant pAL7A7 (containing seven copies of *QUTA*) were investigated further. Transformants pAL7GEA4 and A10, and the R153 wild-type control, were grown with quinic acid or glycerol as carbon source, and the levels of 3-dehydroquinase were determined in cell-free extracts. Transformant pAL7ΔGEA4 was further characterized by measuring the *QUT* enzymes in cell-free extracts of mycelium grown with glucose, glycerol or quinic acid as carbon source. The results of the enzyme assays are shown in Tables 3(a) and 3(b) which show that the *QUT* enzymes are induced in response to quinic acid in a manner indistinguishable from wild-type and there is no elevation of the 3-dehydroquinase enzyme levels, even though there is a presumed increase in the concentration of activator-protein molecules.

These data when taken in conjunction with the previously reported observation that transformants containing multiple copies of *QUTE* but only one copy of *QUTA* (Da Silva *et al.*, 1986) show a wild-type induction pattern for the three *QUT* enzymes, with no elevation of 3-dehydroquinase enzyme levels, lead to the conclusion that concentration of the activator protein is not the sole limiting factor in the expression of the *QUT* cluster. The

data strongly suggest that there are a limited number of binding sites for the activator protein and, when these sites all have activator bound, no further increase in expression occurs, even in the presence of a presumed excess of activator-protein molecules. By increasing the copy number of the *QUTE* gene in the presence of an equal number of *QUTA* (activator) genes, the presumed increased numbers of activator molecules are able to saturate the increased binding sites provided by the *QUTE* genes, leading to selective overexpression of 3-dehydroquinase.

Purification of catabolic 3-dehydroquinase from transformant PAL7A7

As the first stage in the elucidation of the reaction mechanism (K. Kleanthous, J. R. Coggins & A. R. Hawkins, unpublished work) and ultimately to form stable crystals that could be used to deduce the three-dimensional structure of the enzyme, transformant pAL7A7 was used as the starting material for the purification of 3-dehydroquinase. Purification of 3-dehydroquinase from wild-type mycelium grown with quinic acid as carbon source has been reported previously (Hawkins *et al.*, 1982), and essentially the same extraction procedure was followed, except that the harvested mycelium was ground in liquid N₂, the heat step was at 71 °C for 10 min and the Sephadex G-200 chromatography was found to be unnecessary and was omitted. Table 4 shows a typical purification that yields enzyme that is chromatographically pure after silver staining. Fig. 2 shows a Coomassie Blue-stained SDS/PAGE gel monitoring the purification process of one typical enzyme extraction, and including as a control the crude extract from a wild-type strain, R153, grown with quinic acid as carbon source. Inspection of the gel shows that: (1) the final product is chromatographically pure; (2) the product has an apparent molecular mass of 16.98 kDa, which is in good agreement with the deduced *M_r* derived from the DNA sequence; and (3) a band corresponding to the 3-dehydroquinase enzyme is present in the crude extract of transformant pAL7A7 that is not discernible in the equivalent extract from the wild-type strain.

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