Inducible overproduction of the *Aspergillus nidulans* pentafunctional AROM protein and the type-I and -II 3-dehydroquinases from *Salmonella typhi* and *Mycobacterium tuberculosis*

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The *aroQ* gene of *Mycobacterium tuberculosis*, encoding a type-II 3-dehydroquinase, and the *aroD* gene of *Salmonella typhi*, encoding a type-I 3-dehydroquinase, have been highly overexpressed in *Escherichia coli* using the powerful *trc* promoter contained within the expression vector pKK233-2. The *M. tuberculosis* type-II 3-dehydroquinase has been purified in bulk from overproducing strains of *E. coli* to greater than 95% homogeneity. The protein is extremely heat-stable, is active as a homododecamer and has the lowest reported K_m value of any type-II 3-dehydroquinase. The pentafunctional *aromA* gene of *Aspergillus nidulans* has been overexpressed more than 120-fold in an *A. nidulans* *aromA*[−] *qutB*[−] double mutant from a truncated quinate-inducible *qutE* promoter, such that the AROM protein is visible as a significant fraction (approx. 6%) in cell-free crude extracts. The *M. tuberculosis* *aroQ* gene has been fused to the same truncated *qutE* promoter and shown to encode quinate-inducible 3-dehydroquinase activity that allows a *qutE*[−] mutant strain of *A. nidulans* to utilize quinate as sole carbon source.

INTRODUCTION

In filamentous fungi, 3-dehydroquinase (DHQ) and dehydroshikimate (DHS) are intermediates in the shikimate and quinate pathways, both of which have been most extensively characterized in *Aspergillus nidulans* and *Neurospora crassa* (see Scheme 1) (Hawkins *et al.*, 1982, 1985, 1988, 1992; Beri *et al.*, 1987; Whittington *et al.*, 1987; Grant *et al.*, 1988; Geever *et al.*, 1989; Lamb *et al.*, 1990, 1991, 1992a,b). In *A. nidulans*, steps 2–6 in the shikimate pathway are catalysed by the constitutively produced pentafunctional protein product encoded by the complex *aromA* gene (Kinghorn & Hawkins, 1982; Charles *et al.*, 1985, 1986). Similar fused polypeptides have been described in *N. crassa* (Coggins *et al.*, 1987) and *Saccharomyces cerevisiae* (Duncan *et al.*, 1987). In prokaryotes the corresponding five reactions are catalysed by constitutively produced monofunctional enzymes which are not required to aggregate for activity (Millar & Coggins, 1986; Anton & Coggins, 1988; Millar *et al.*, 1986; Duncan *et al.*, 1984, 1986). Evidence from DNA sequence analysis strongly suggests that the pentafunctional product of the *aromA* gene has evolved by a series of gene fusions from the ancestral sequences corresponding to the five separate genes present in prokaryotes (Hawkins, 1987). With the aid of site-directed mutagenesis, Hawkins & Smith (1991) have demonstrated that discrete portions of the AROM polypeptide can fold and function in *Escherichia coli* as monofunctional enzymes, thereby lending support to the theory of gene fusion. In addition, the DHQ synthase domain of the AROM polypeptide has been overexpressed in *E. coli* and purified to greater than 80% homogeneity by a simple two-step procedure (van den Hombergh *et al.*, 1992). In filamentous fungi, the enzymes catalysing reactions including the shared substrates DHQ and

DHS have a complex evolutionary relationship: (a) the quinate dehydrogenase enzyme (quinat:NAD oxidoreductase; EC 1.1.1.24) of the quinate-utilization pathway and the shikimate dehydrogenase enzyme (EC 1.1.1.25) of the shikimate pathway have been found to show extensive homology at the protein sequence level (Hawkins *et al.*, 1988), but conversely (b) the comparison of the protein sequences of the biosynthetic 3-dehydroquinase (DHQ hydro-lyase; EC 4.2.1.19) of the shikimate pathway and the catabolic 3-dehydroquinase of the quinate-utilization pathway has led to the proposal that the two enzymes have arisen by convergent evolution (Hawkins, 1987; Da Silva *et al.*, 1986).

3-Dehydroquinase enzymes comprise two distinct groupings: one, typified by the biosynthetic dehydroquinase of *E. coli*, is active as a dimer with a subunit molecular mass of 28 kDa and a reaction mechanism that proceeds via a Schiff's-base intermediate (Chaudhuri *et al.*, 1991); the second class, typified by the catabolic 3-dehydroquinase of *A. nidulans*, have subunit molecular masses of 14–16 kDa, but are active as homododecamers and the reaction mechanism does not appear to proceed via a Schiff's-base intermediate. The division of the 3-dehydroquinases into type-I and type-II classes has been suggested by White *et al.* (1990), and Garbe *et al.* (1991) have proposed that the two classes of 3-dehydroquinases are mechanistically, as well as phylogenetically, distinct. Until recently, biosynthetic 3-dehydroquinases have been found to be type I in phylogeny, whereas the only examples of type-II 3-dehydroquinases were found in the catabolic quinate-utilization pathway. More recently type-II 3-dehydroquinases have been found in the Gram-positive bacteria *Mycobacterium tuberculosis* and *Streptomyces coelicolor* (Garbe *et al.*, 1991; White *et al.*, 1990). A gene, designated *aroQ*, encoding the 3-dehydroquinase from *M. tuberculosis* was re-

Abbreviations used: AROM, the protein encoded by the *aromA* gene; *aromA*, the gene encoding the pentafunctional AROM protein; DHQ, 3-dehydroquinase; DHS, dehydroshikimate; IPTG, isopropyl β -thiogalactoside.

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Scheme 1. The genes, enzymes and metabolites comprising the shikimate and quinate pathways in *A. nidulans*

This is essentially the same as Scheme 1 of Lamb *et al.* (1992b), but is included here for a better understanding of the pathways involved.

peatedly cloned by complementation of an *aroD* mutation in *E. coli* (Garbe *et al.*, 1991), suggesting that it is the only gene capable of encoding a 3-dehydroquinase in *M. tuberculosis*. Furthermore, it is very closely linked (initiation codon overlapping stop codon) to the gene encoding DHQ synthase in *M. tuberculosis*; thus there is strong evidence for the assertion that the type-II 3-dehydroquinase in *M. tuberculosis* has a biosynthetic role.

Garbe *et al.* (1991) have detected sequence similarity at the protein level which suggests that the type-II dehydroquinases and the DHQ synthases share a common ancestor, a possible case of retro-evolution (Horowitz, 1965). As part of a project to analyse the three-dimensional structure of (a) the pentafunctional AROM protein, (b) a family of type-I and type-II 3-dehydroquinases and (c) DHQ synthases, by solving their crystal structures by using X-ray diffraction, we have overexpressed the *M. tuberculosis* type-II 3-dehydroquinase and the *S. typhi* type-I 3-dehydroquinase in *E. coli* to facilitate their purification. The pentafunctional AROM protein of *A. nidulans* (which contains a type-I 3-dehydroquinase) has proved refractory to overproduction in *E. coli* (Hawkins & Smith, 1991), therefore we have constructed an *A. nidulans* strain capable of supporting quinate-inducible overproduction.

MATERIALS AND METHODS

A. nidulans strain 1179 was constructed by crossing strain R46 *aromA1103* (Roberts, 1968) with R21 *qutB42* and screening individual progeny in growth tests for their ability to use quinate as carbon source and ability to grow in the absence of aromatic amino acid supplementation. One isolate designated 1179, which had the appropriate phenotype of quinate non-utilization and inability to grow in the absence of aromatic amino acids, was chosen as the host for transformation experiments after confirmation by enzyme assay that the strain lacked quinate dehydrogenase. The genotypes of the other *A. nidulans* strains and the plasmids used in this work are summarized in Table 1. The origins of the *E. coli* strains used in this work together with the media for the growth and propagation of *E. coli* and *A. nidulans* have been described previously (Willett *et al.*, 1969;

Hawkins *et al.*, 1984; Armitt *et al.*, 1976). Materials were obtained as described in Charles *et al.* (1985). The plasmid pKK233-2 was from Pharmacia and the phagemid pBluescript II was from Stratagene (Cambridge, U.K.).

Standard methods

Recombinant DNA techniques were as previously described (Maniatis *et al.*, 1982). Southern-blot analysis was performed by the method of Jeffreys *et al.* (1980) and DNA restriction fragments were purified by the 'freeze squeeze' method of Tautz & Renz (1982). The preparation of cell-free extracts and dehydroquinase, DHS dehydratase, quinate dehydrogenase and

Table 1. Description of strains and recombinant plasmids used in this work

<i>A. nidulans</i>	Description
WA53	<i>wA3, pyrG189, qutE208, pyroA4</i>
R153	<i>wA3, pyroA4</i>
R21	<i>yA2, pabaA1</i>
R44	<i>wA3, ribo1, adl4</i>
QAT12	<i>wA3, pro1, pyroA4</i> transformed with pNUCF2
QAT15	<i>wA3, pro1, pyroA4</i> transformed with pNUFC2
E208	<i>yA2, pabaA1, qutE208</i>
1300-3	<i>yA2, pabaA1</i> transformed with pNUCF5
1304-7	<i>yA2, pabaA1</i> transformed with pNUFC50

Plasmid	Expression in organism	Promoter	Coding sequence	Termination signals
pKK233-2	<i>E. coli</i>	P <i>trc</i>	—	<i>rrnBT₁T₂</i>
pKK9	<i>E. coli</i>	P <i>trc</i>	<i>qutE</i>	<i>rrnBT₁T₂</i>
pKK44	<i>E. coli</i>	P <i>trc</i>	<i>aroQ</i>	<i>aroQ</i>
pKK45	<i>E. coli</i>	P <i>trc</i>	<i>aroD</i>	<i>rrnBT₁T₂</i>
pBS- <i>aroQ</i>	—	—	<i>aroQ</i>	<i>aroQ</i>
pNUFC2	<i>A. nidulans</i>	<i>qutE(NcoI)</i>	<i>aromA</i>	<i>aromA</i>
pNUFC5	<i>A. nidulans</i>	<i>qutE(NcoI)</i>	<i>qutE</i>	<i>qutE</i>
pNUFC50	<i>A. nidulans</i>	<i>qutE(NcoI)</i>	<i>aroQ</i>	<i>aroQ*</i>

* Prokaryotic termination signal unlikely to function efficiently in *A. nidulans*.

shikimate dehydrogenase assays were performed as described previously (Hawkins *et al.*, 1984; Coggins *et al.*, 1987). Protein determination was by the method of Lowry *et al.* (1951), with BSA as standard.

Purification of the type-II dehydroquinases

Luria broth (10 litres) supplemented with 100 mg of ampicillin/l was inoculated with a starter culture of *E. coli* strain JM105 harbouring either plasmid pKK9 or pKK44 (see the Results and discussion section). The cultures were incubated with constant agitation at 37 °C until the cells had grown to A_{550} 0.2. To induce transcription from the *trc* promoter, isopropyl β -thiogalactoside (IPTG) was then added to a concentration of 200 mg/l, and the cultures were incubated for a further 8 h. Cells were then harvested by centrifugation in a Beckman JA-14 rotor at 8000 rev./min for 10 min, yielding approx. 40 g (wet wt.) of *E. coli* cells, which were subsequently lysed by sonication, and the type-II dehydroquinases were purified essentially as previously described (Hawkins *et al.* 1982).

PAGE

Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970), with a 5% (w/v) polyacrylamide stacking gel and a 12% (w/v) polyacrylamide separating gel. Gels were stained for protein with Coomassie Blue.

PCR amplification

PCR reactions were carried out with a Gene Amp Kit from Perkin-Elmer Cetus by following the manufacturer's general recommended reaction conditions, in combination with a Biometra Trio Thermoblock. Individual reaction conditions were modified according to the following protocol: (1) for 30-mer oligonucleotides containing up to six mismatched bases, initial DNA denaturation was at 94 °C for 1.5 min, and annealing was at 50 °C for 1 min; extension took place at 72 °C for 1 min and subsequent denaturation was at 94 °C for 1 min; the cycle was repeated 24 times.

In each case approx. 2 ng of template DNA was used in conjunction with appropriate primers at an approximate concentration of 0.15 μ M each. The reactions were carried out in a final volume of 100 μ l, and the efficiency of amplification was monitored by electrophoresing 10 μ l of the final reaction mixture through a 0.8% (w/v) agarose gel and staining with ethidium bromide. In each case, a very strong signal of the predicted molecular mass was observed, with no additional bands or any evidence of degradation as monitored by a complete lack of 'smearing' of the final PCR product. After electrophoretic analysis, the remaining 90% of the PCR product was subjected to phenol extraction, ethanol precipitation and, after drying, digestion with the restriction endonucleases *Bsp*HI and *Hind*III. Digestion with the two restriction enzymes was possible, as the oligonucleotides used for the PCR reaction were designed to provide a sequence of at least 7 bp beyond the enzyme-recognition site.

IPTG-inducible gene overexpression

IPTG-induced gene overexpression was initially screened for by inoculating individual colonies containing recombinant pKK233-2 or unmodified pKK233-2 (control) plasmids into 2 ml of Luria broth containing 100 μ g of ampicillin/ml and 0.2 mg of IPTG/ml. Cells were then grown with vigorous agitation for 12–14 h, harvested by centrifugation, and lysed in 100 μ l of SDS/PAGE loading buffer by heating in a boiling-water bath for 5 min. Approx. 0.1 A_{550} unit of cells was subjected to SDS/PAGE with appropriate markers, and the proteins were stained with Coomassie Brilliant Blue. Plasmids from

overexpressing strains detected by this screening procedure were then transformed into strain *E. coli* JM105 which contains the *LacI*^q mutation, allowing more controlled IPTG-inducible gene overexpression. Strains of JM105 containing recombinant pKK233-2 or unmodified (control) plasmids were initially grown in Luria broth supplemented with 100 μ g of ampicillin/ml at 37 °C, and then used to seed a second culture of drug-supplemented Luria broth to an A_{550} of 0.05. This culture was then aerated vigorously at 37 °C until an A_{550} of 0.2 was reached, when 0.2 mg of IPTG/ml was added, and the culture incubated for a further 8 h. A 1.0 A_{550} equivalent of cells was taken at zero time and at 8-h intervals after that, and 0.1 A_{550} equivalent of cells was subjected to SDS/PAGE as described above.

Sonicated cell-free extracts of *E. coli* were prepared for enzyme assay as described previously (Hawkins & Smith, 1991), in a buffer comprising 50 mM-potassium phosphate, 1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride (buffer A).

RESULTS AND DISCUSSION

Subcloning the *M. tuberculosis aroQ* gene and the *S. typhi aroD* genes into the expression vector pKK233-2

The *M. tuberculosis aroQ* gene encoding a type-II 3-dehydroquinase and the *S. typhi aroD* gene encoding a type-I 3-dehydroquinase have been isolated previously by complementation of an *aroD*⁻ mutant strain of *E. coli*, and their DNA sequences reported (Garbe *et al.*, 1991; Servos *et al.*, 1991a). An *M. tuberculosis aroQ* expression cassette was generated by ligating a 1.05 kb *Bcl*I fragment of the *aroQ* gene into plasmid pSI3 (Servos *et al.*, 1991b) to generate pBS *aroQ*. This 1.05 kb *BC*II fragment lacks the first eight codons of the structural gene for *aroQ*. The missing 24 bp are supplied by pSI3, which also contains an in-frame ATG codon that is part of a *Bsp*HI site and a *Hind*III site at the 3' end of the gene.

The fragment containing the *aroQ* gene was released from pBS *aroQ* by digestion with *Bsp*HI and *Hind*III, after amplification of the DNA in the *dam* methylase-deficient *E. coli* strain BRD511. The *Bsp*HI–*Hind*III fragment was ligated into the *Nco*I–*Hind*III-digested expression vector pKK233-2 to yield the *aroQ* expression plasmid pKK44.

On the basis of the published sequence (Servos *et al.*, 1991a), two oligonucleotides, one a 25-mer which introduced a *Bsp*HI site in frame with the AUG translational start codon and one a 35-mer that introduced a *Hind*III site at the 3' end, were used to PCR-amplify the *S. typhi aroD* gene. After successful amplification, the DNA fragment was digested with *Bsp*HI and *Hind*III and ligated into the *Nco*I–*Hind*III-digested vector pKK233-2 to yield the plasmid pKK45. Ligation mixtures used to generate plasmids pKK44 and pKK45 were used to transform *E. coli* strain SK3430 (*aroD*⁻; lacking 3-dehydroquinase) to ampicillin-resistance on rich medium. Individual colonies were picked off into broth supplemented with ampicillin and IPTG, and the resultant cells were screened for enzyme overproduction as described in the Materials and methods section. The plasmid from one each of the two types of *E. coli* overproducing transformant was purified and used to transform *E. coli* strain JM105, which contains the *lacI*^q mutation, thereby allowing a more controlled IPTG-inducible enzyme overproduction. Experimental strains containing plasmids pKK44 and pKK45 and a control strain with unmodified pKK233-2 plasmid, were subjected to an 8 h induction with IPTG, and cells collected at 1 h intervals during the induction period were screened for protein by SDS/PAGE as described in the Materials and methods section. Figs. 1(a), 1(b) and 1(c) show photographs of the

Fig. 1. SDS/PAGE analysis of *E. coli* strains overproducing the *S. typhi* type-I and the *M. tuberculosis* type-II 3-dehydroquinases

E. coli JM105 strains overproducing (a) the *S. typhi* type-I 3-dehydroquinase, (b) the *M. tuberculosis* type-II 3-dehydroquinase or (c) control non-overexpressing strain; 0.1 *A*₅₅₀ unit of *E. coli* cells at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 23 h after addition of IPTG were boiled in SDS/PAGE loading buffer, then loaded and subjected to electrophoresis. The SDS/PAGE was standardized by electrophoresing protein molecular-mass markers (shown in outside lanes), comprising phosphorylase *b* (97 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18 kDa) and lysozyme (14 kDa). The positions of the overproduced 3-dehydroquinase enzymes are marked (o).

Table 2. Purification scheme for the type-II dehydroquinases of *M. tuberculosis* and *A. nidulans* expressed in *E. coli*

The results presented are from a typical purification starting from 40 g of *E. coli* cells, and enzyme units are μ mol of product/min per mg at 37 °C.

Step	Volume	Concn. of protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield
<i>M. tuberculosis</i> 3-dehydroquinase								
1 Crude extract	385	3.61	1390	19.6	7540	5.43	1.0	100
2 Heat shock, 72 °C	650	0.59	384	13.0	8420	21.9	4.0	111
3 30–55 %-satn. (NH ₄) ₂ SO ₄	15.7	18.0	282	584	9170	32.5	6.0	122
4 Sephacryl S300HR	186	1.15	214	23.6	4380	20.5	3.8	58
5 DEAE-cellulose DE-52	145	0.56	81	24.2	3510	43.3	8.0	47
<i>A. nidulans</i> catabolic 3-dehydroquinase								
1 Crude extract	400	3.29	1316	110	44200	33.6	1.0	100
2 Heat shock, 72 °C	380	0.75	285	107	40500	142	4.2	92
3 30–55 %-satn. (NH ₄) ₂ SO ₄	15	6.00	90	2500	37500	417	12.4	85
4 Sephacryl S300HR	60	0.30	18	278	16700	926	27.6	38
5 DEAE-cellulose DE-52	22	0.21	4.6	270	5900	1284	38.2	13

induction profile for *E. coli* strains containing the pKK44 and pKK45 and unmodified pKK233-2. Both proteins were overproduced at a very high level, amounting to 50% of total cell protein in the case of *S. typhi* type-I 3-dehydroquinase. Cell-free sonicated extracts taken from the 8 h induction point were assayed for 3-dehydroquinase activity to check that the overproduced protein was soluble and functional. The strain containing the *S. typhi* type-I 3-dehydroquinase had a specific activity of 77 units/mg, and the strain containing the *M. tuberculosis* type-II 3-dehydroquinase had a specific activity of 5.4 units/mg, demonstrating that the enzymes were soluble and functional in *E. coli*.

Purification of type-II 3-dehydroquinases from overproducing *E. coli* strains

The type-II 3-dehydroquinase of *M. tuberculosis* was purified from *E. coli* strain JM105 harbouring the plasmid pKK44, and as a control the type-II (catabolic) 3-dehydroquinase of *A. nidulans* was purified from the *E. coli* strain TG1 harbouring plasmid pKK9. The two enzymes were purified essentially as described by Hawkins *et al.* (1982), and a purification scheme is shown in Table 2 with a photograph showing the purity at each

stage in Fig. 2. In both cases the 3-dehydroquinases comprised a major band in the crude extract of soluble protein. The heat-shock and (NH₄)₂SO₄ precipitation purified both of the proteins to near-homogeneity, whereas the gel-filtration chromatography step conferred only a small increase in specific activity, and with the *A. nidulans* 3-dehydroquinase resulted in large overall losses. After the ion-exchange chromatography step, both of the 3-dehydroquinases were judged greater than 95% pure on the basis of Coomassie Blue staining, but this step also resulted in significant losses, especially with the *A. nidulans* enzyme. In the work reported here, only 13% of the original activity of the *A. nidulans* type-II 3-dehydroquinase was recovered, in contrast with the 61% recovered (from an overexpressing strain of *A. nidulans*) by a similar method (omitting the gel-filtration chromatography step) by Beri *et al.* (1990). Hautala *et al.* (1975) have reported that the *N. crassa* type-II 3-dehydroquinase forms inactive very-high-molecular-mass aggregates at high 3-dehydroquinase concentrations. It seems likely therefore that the depressed yield of *A. nidulans* 3-dehydroquinase reported here relative to that achieved by Beri *et al.* (1990) is due to aggregation of the 3-dehydroquinase when resuspended in a minimum volume before the chromatography steps. Beri *et al.* (1990) diluted the

Fig. 2. SDS/PAGE demonstrating the purification of the type-II dehydroquinases of *M. tuberculosis* and *A. nidulans* from overproducing *E. coli* strains

Samples of equal dehydroquinase activity of (a) *M. tuberculosis* and (b) *A. nidulans* type-II dehydroquinase-overproducing strains at each stage of the purification process were analysed by SDS/PAGE. Lane 1, crude extract; lane 2, after 70 °C heat shock; lane 3, $(\text{NH}_4)_2\text{SO}_4$ precipitate (30–55 % saturation); lane 4, after S-300HR gel-filtration chromatography; lane 5, after DE-52 DEAE-cellulose ion-exchange chromatography. The SDS/PAGE was standardized by electrophoresing protein molecular-mass markers (shown in outside lanes), comprising myosin (205 kDa), β -galactosidase (116 kDa), phosphatase (92 kDa), BSA (67 kDa), alcohol dehydrogenase (41 kDa), chymotrypsinogen A (25 kDa), cytochrome *c* (12 kDa).

30–55 %-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate to a large volume [in order to decrease the ionic strength of the buffer due to residual $(\text{NH}_4)_2\text{SO}_4$ in the pellet] before loading on to the ion-exchange column, and so their method should be followed to alleviate the substantial losses that this aggregation phenomenon causes.

During the gel-filtration step of the purification of the *M. tuberculosis* 3-dehydroquinase, a very interesting phenomenon was observed. The A_{280} profile showed two clear peaks separated by a trough, whereas the 3-dehydroquinase activity was initially found to be localized in the first absorbance peak with a very pronounced shoulder in the region of the second absorbance peak. However, after incubation at 4 °C for 3 days, the second absorbance peak was now found to contain at least as much dehydroquinase activity as the first. Small volumes of various fractions from this elution profile were run on a denaturing SDS/12.5 % polyacrylamide gel and the lanes corresponding to both peaks were found to contain the same protein composed of a 14 kDa subunit. The separation on the basis of size used in gel-filtration chromatography suggests that the explanation for this phenomenon involves at least two forms of the *M. tuberculosis* type-II dehydroquinase in dynamic equilibrium, possibly an enzymically active homododecamer native form and an inactive monomeric or dimeric form. That the two putative forms could be separated on a gel-filtration column eluted overnight and still had not reached equilibrium the next day strongly suggests that the rate of exchange between the two forms is low. The exact nature of this phenomenon briefly described here (which is not seen during purification of the *A. nidulans* type-II 3-dehydroquinase) requires further investigation. The volume of the Sephacryl S300 column used for the gel filtration was 450 ml, and the leading peaks of enzyme activity for the *M. tuberculosis* and *A. nidulans* extracts were co-eluted to within 10 ml, strongly implying that they have a closely similar native molecular mass. The comparative purification for the *M. tuberculosis* and *A. nidulans* 3-dehydroquinases is shown in Table 2.

The data discussed above show that the type-II 3-dehydroquinases from *A. nidulans* and *M. tuberculosis* have closely similar physical characteristics in terms of native molecular mass and heat-stability. In general, 3-dehydroquinases that have a catabolic role are type II in nature and have relatively high K_m values, i.e. that for *A. nidulans* type-II 3-dehydroquinase

is 150 μM and that for the *N. crassa* type-II 3-dehydroquinase is 360 μM , whereas biosynthetic 3-dehydroquinases tend to be type I in nature, with relatively low K_m values, i.e. that for *N. crassa* AROM type-I 3-dehydroquinase is 5 μM and that for the *E. coli* type-I 3-dehydroquinase is 18 μM . As the *M. tuberculosis* type-II 3-dehydroquinase has a strongly inferred biosynthetic role, the K_m value was determined with the *A. nidulans* type-II 3-dehydroquinase as a control to see if the experimentally deduced value fitted the general pattern for biosynthetic 3-dehydroquinase. The apparent K_m value for the *M. tuberculosis* type-II 3-dehydroquinase was 64 μM and the value for the *A. nidulans* type-II 3-dehydroquinase was 140 μM , which is in close agreement with the value for *A. nidulans* (150 μM) quoted by Kleanthous *et al.* (1992). These observations are consistent with the view that the *M. tuberculosis* type-II 3-dehydroquinase has a biosynthetic role *in vivo*, as it has the lowest reported apparent K_m value for any type-II 3-dehydroquinase.

Quinate-inducible expression from the *qutE* promoter

A 158 bp promoter sequence from the *qutE* gene in plasmid pNUFC2 has previously been shown to direct quinate-inducible expression of the *aroA* locus of *A. nidulans* (Lamb *et al.*, 1991); therefore, in order to determine the utility of this promoter for the systematic overexpression of heterologous genes, a detailed analysis of the functioning of the truncated promoter was initiated. This truncated *qutE* promoter contains putative UAS_{QUT} and CAAT motifs predicted to be involved in the molecular control of transcription, but lacks a 22 bp motif previously identified as possibly being essential for correct transcriptional regulation (Hawkins *et al.*, 1988). As we wished to express the *aroQ* and *aroD* genes of *M. tuberculosis* and *S. typhi* in *A. nidulans* and *E. coli*, using the *qutE* and *trc* promoters respectively, we constructed plasmid pNUFC5, which has restriction sites that are compatible with pKK233-2.

A sonicated fragment of DNA containing the *qutE* gene subcloned into M13 MP9 RF DNA and designated clone 43 has been described previously (Da Silva *et al.*, 1986). A mutant derivative of this clone designated M6 and containing an *NcoI* site engineered into the sequence containing the AUG translational start codon was described by Lamb *et al.* (1991) and Hawkins & Smith (1991). The entire insert of *A. nidulans*

Table 3. Specific activities of quinate-utilization enzymes in *qutE208*/pNUFC5 and pNUFC50 transformants

Conidiospores (2×10^8) were inoculated into 200 ml of minimal medium supplemented with uracil, *p*-aminobenzoic acid and glucose as carbon source. The cultures were grown with vigorous aeration at 37 °C for 17 h, the mycelium harvested by filtration, and divided into two equal portions, which were placed in 200 ml of minimal medium supplemented with uracil, *p*-aminobenzoic acid with either glucose (0.2 %, w/v) or quinate (0.1 %, w/v) as carbon source. After a 5 h induction at 37 °C, cell-free extracts were assayed for quinate pathway enzymes, and are shown as specific activities and relative specific activities compared with the R21 control.

Strain	Transforming plasmid	Specific activities (μmol of product/min per mg of protein)		
		Quinate dehydrogenase	Dehydroquinase	Dehydroshikimate dehydratase
R21	—	0.34 (1.0)	0.38 (1.0)	0.15 (1.0)
E208	—	0.31 (0.9)	nd* (0.0)	0.24 (1.6)
1300	pNUFC5	0.24 (0.7)	1.76 (4.7)	0.14 (0.9)
1301	pNUFC5	0.28 (0.8)	0.48 (1.3)	0.16 (1.0)
1304	pNUFC50	0.26 (0.8)	0.04 (0.1)	0.13 (0.8)
1305	pNUFC50	0.21 (0.6)	0.04 (0.1)	0.14 (0.9)
1307	pNUFC50	0.37 (1.1)	0.04 (0.1)	0.14 (0.9)

*nd, not detectable.

DNA in clone M6 was purified from a low-melting-point agarose gel after digestion with *Sac*I and *Hind*III (both of which cut in the multiple cloning site of M13 MP9), and ligated into *Sac*I- and *Hind*III-digested pUC18 (from which the unique *Eco*RI site had previously been deleted) DNA (Lamb *et al.*, 1991) to yield the recombinant plasmid pNUFC5, a procedure that changes the final nucleotide in the *qutE* promoter from an A to a C. Digestion of pNUFC5 with *Nco*I and *Hind*III removes the *qutE* gene and its 3' polyadenylation signal, allowing heterologous genes to be fused directly to the truncated *qutE* promoter.

Plasmid pNUFC50 was constructed by isolating the *M. tuberculosis aroQ* coding sequence fragment from a *Bsp*HI–*Hind*III digest of pBS-*aroQ* and ligating it to *Nco*I–*Hind*III-digested pNUFC5 (*Bsp*HI has sticky ends compatible with *Nco*I). This places the *aroQ* gene under the control of the *qutE* promoter, although in this plasmid construct transcriptional termination of *aroQ* message can only be accomplished by the *aroQ* termination signals.

Protoplasts of the strain R21*qutE208* were transformed with 20 μg of either pNUFC5 or pNUFC50 DNA by the method of Ballance *et al.* (1983), and transformants were selected for the ability to grow on quinate as sole carbon source. A total of 16 transformants were isolated from the transformation of R21*qutE208* with pNUFC5 DNA, but only five were recovered from the pNUFC50 DNA transformation. Growth tests with four of the pNUFC5 (*qutE*) transformants designated 1300, 1301, 1302 and 1303, along with four of the pNUFC50 (*aroQ*) transformants designated 1304, 1305, 1306 and 1307 and controls of R21 (*y*, *pabaA1*) and R21*qutE208* (*y*; *pabaA1*; *qutE208*) were carried out and confirmed that the transformants isolated were able to grow on quinate as sole carbon source. There was a range of growth responses on quinate, with some of the *qutE* transformants growing as well as the wild-type and some markedly worse, but none of the *aroQ* transformants were able to grow as well as the wild-type.

Conidiospores from the strains R21, R21*qutE208*, 1300, 1301, 1304, 1305 and 1307 were isolated and the activities of the quinate-utilization enzymes in the transformants determined (see Table 3). Inspection of Table 3 leads to the following conclusions: (a) the relative specific activities of the quinate dehydrogenase and DHS dehydratase were similar in the wild-type R21, the recipient *qutE* mutant (R21*qutE208*) and all the transformants, demonstrating that the transformation of the pNUFC5 and

pNUFC50 plasmids into *A. nidulans* did not disrupt the functional integrity of the other *qut* genes; (b) the relative specific activity of type-II dehydroquinases in the transformants did show significant variation from the wild-type. With the *qutE* transformants, one had a 4–5-fold higher specific activity and one had a specific activity very similar to the wild-type, whereas the three *aroQ* transformants all had specific activities 8–10-fold lower than the wild-type. It was noted that the *aroQ* transformants with the decreased dehydroquinase activity showed a less vigorous growth response on quinate as sole carbon source than did the *qutE* transformants 1300 and 1301.

A Southern blot was performed on *Eco*RI-digested chromosomal DNA isolated from the transformants using [α - ^{32}P]dCTP-labelled pBR322 DNA as a probe for the vector sequences expected to be present in the *A. nidulans* chromosomal DNA in some types of transformants. Inspection of the autoradiograph showed that transformants 1300 and 1301 probably contained one integrated copy of pNUFC5, but in contrast the three pNUFC50 transformants, 1304, 1305 and 1307, contained multiple tandem integrations of the plasmid carrying the *aroQ* gene under the control of the *qutE* promoter (results not shown). These results strongly suggest that 5–10-fold overexpression of the catabolic dehydroquinase in *A. nidulans* is possible from one or two functional copies of the *qutE* gene integrated at non-homologous sites. However, a much larger number of integrated copies of the *qutE*-promoter-driven *aroQ* gene encoded by pNUFC50 are required for complementation of the *qutE* mutation.

This last result initially suggests that the *M. tuberculosis* type-II 3-dehydroquinase from the shikimate pathway is unsuited for the catabolic role required to complement the *qutE* mutation. However, the inefficiency of the pNUFC50 transformants in complementation of the *qutE* mutation is more likely to be due to poor expression, as the construct uses the transcription termination signal from the *M. tuberculosis* gene. The *aroQ* gene therefore has no facility for appropriate termination with a fungal signal; thus the stability of the transcribed mRNA is likely to be substantially decreased, which will limit the production of the translated product. The *qutE208* mutation used in these transformation experiments is very stable, but its nature has not been determined; therefore it is possible that it may be a mis-sense mutation producing a stable but inactive monomeric subunit. Alternatively, therefore, the poor growth of *aroQ*

Table 4. Effect of carbon catabolite repression on protein production under the control of the *qutE* promoter in transformed strains

Conidiospores (2×10^8) were inoculated into 200 ml of minimal medium supplemented with either (a) *p*-aminobenzoic acid or (b) riboflavin and adenine with (0.4 %, w/v) glucose as carbon source. The cultures were incubated with vigorous aeration at 37 °C for 17 h. The mycelium was harvested by filtration and divided into three (a) or four (b) equal amounts which were transferred to fresh minimal medium (200 ml) with a range of carbon sources, i.e. (a) 0.2 % glucose, 0.2 % glucose + 0.1 % quinate, 0.1 % quinate; (b) 0.2 % glucose, 0.2 % glycerol + 0.2 % quinate, 0.2 % glucose + 0.2 % quinate, 0.2 % quinate and incubated at 37 °C with vigorous aeration for a further 5 h. The mycelium was again harvested by filtration and cell-free extracts were prepared. Biosynthetic (type-I) dehydroquinase activity was determined by assaying total dehydroquinase activity and subtracting the heat-stable (after 10 min at 70 °C) fraction, which represents the catabolic (type-II) dehydroquinase activity. In the first experiment (a), specific activities are listed for the three quinate-utilization enzymes in the strains tested for inducing (quininate) and repressing (quininate + glucose) conditions, with the values in parentheses representing the fraction of activity present in repressing conditions compared with inducing conditions. In the second experiment (b) the specific activities of the two constituent enzymes of the AROM complex that were assayed, biosynthetic dehydroquinase and shikimate dehydrogenase, are shown for the strains R44, QAT12 and QAT15 along with the catabolic dehydroquinase activity in the wild-type strain R44. The values in parentheses represent the fraction of activity present in the various conditions with respect to induction by quinate alone.

(a)			Specific activities (μmol of product/min per mg of protein)		
Strain	Transforming plasmid	Medium	Quinate dehydrogenase	3-Dehydroquinase	DHS dehydratase
R21	–	Quinate	0.39 (1.0)	0.31 (1.0)	0.18 (1.0)
		Quinate + glucose	0.20 (0.5)	0.06 (0.2)	0.08 (0.5)
1300	pNUFC5	Quinate	0.23 (1.0)	1.49 (1.0)	0.18 (1.0)
		Quinate + glucose	0.19 (0.8)	0.31 (0.2)	0.08 (0.5)
1304	pNUFC50	Quinate	0.29 (1.0)	0.05 (1.0)	0.13 (1.0)
		Quinate + glucose	0.07 (0.3)	0.02 (0.4)	0.05 (0.4)
(b)			Specific activities (μmol of product/min per mg of protein)		
Strain	Transforming plasmid	Medium	Catabolic 3-dehydroquinase	Biosynthetic 3-dehydroquinase	Shikimate dehydrogenase
R44	–	Quinate	0.40 (1.0)	0.009	0.012
		Quinate + glycerol	0.22 (0.6)	0.016	0.017
		Quinate + glucose	0.04 (0.1)	0.010	0.016
		Glucose	nd (0)	0.011	0.007
QAT12	pNUFC2	Quinate	–	0.11 (1.0)	0.18 (1.0)
		Quinate + glycerol	–	0.19 (1.7)	0.26 (1.4)
		Quinate + glucose	–	0.08 (0.7)	0.12 (0.7)
		Glucose	–	0.01 (0.1)	0.01 (0.06)
QAT15	pNUFC2	Quinate	–	0.15 (1.0)	0.19 (1.0)
		Quinate + glycerol	–	0.20 (1.3)	0.28 (1.5)
		Quinate + glucose	–	0.05 (0.3)	0.07 (0.4)
		Glucose	–	0.01 (0.1)	0.01 (0.05)

transformants may be due to formation of hetero-oligomeric native forms of 3-dehydroquinase *in vivo* using *qutE208*- and *aroQ*-encoded subunits.

Effect of catabolite repression on the expression of a range of proteins driven by the truncated *qutE* promoter

To test that the regulation of the 158 bp truncated *qutE* promoter was unchanged from the wild-type, the effect of carbon catabolite repression on the production of proteins encoded by sequences placed under the control of the *qutE* promoter was determined. Carbon catabolite repression was examined in two wild-type strains (R153 and R44), the *qutE*-overexpressing strain 1300, the *aroQ*-expressing strain 1304 and two strains overexpressing the complex *aromaA* gene, QAT12 and QAT15 (Lamb *et al.*, 1991) (see Tables 4a and 4b). Strains QAT12 and QAT15 have the *aromaA* locus fused to the truncated *qutE* promoter and overproduce the AROM protein on induction with quinate. Conidiospores were grown with glucose as carbon source, subsequently shifted to medium containing glucose, quinate or glucose and quinate, and cell-free extracts were assayed for the appropriate enzyme activities, which are shown in Table 4. In all the transformants tested, the regulation of

production of the proteins under the control of the truncated *qutE* promoter was indistinguishable from that of the wild-type strains, suggesting that the putative 22-nucleotide motif present in some of the 5' untranslated regions of the *qut* cluster, including the *qutE* gene (Hawkins *et al.*, 1988), is not essential for either high-level transcription or correct regulation.

Quinate-inducible overproduction of the *A. nidulans* AROM protein

Having shown that the truncated *qutE* promoter functions in a manner indistinguishable from the wild-type, and was therefore potentially of use to drive the transcription of any heterologous gene fused to it, we addressed the problem of enhancing the transcription levels. All the strains used in the transformations reported above and previously (Lamb *et al.*, 1991) which used the truncated *qutE* promoter had a wild-type functional *qutB*⁺ gene encoding quinate dehydrogenase. This meant that, in all these transformants, quinate was actively metabolized to protocatechuate, thereby possibly lessening the efficiency of quinate as an inducer for genes driven by the *qutE* truncated promoter. In order to obviate this potential problem, we constructed an *aromaA*[−]/*qutB*[−] double mutant (designated strain

Table 5. Specific activities of biosynthesis (type-I) 3-dehydroquinase and shikimate dehydrogenase in AROM-overproducing strains compared with the wild-type R153

Conidiospores (2×10^8) were used to inoculate 200 ml of minimal medium supplemented with pyridoxine, *p*-aminobenzoic acid, riboflavin and adenine and 0.4% (w/v) glucose as carbon source. After incubation at 37 °C with vigorous aeration for 18 h, the mycelium was harvested by filtration and the mycelial mat was split into two equal portions. The portions were placed in 200 ml of minimal medium supplemented as above with either glucose (0.2%, w/v) (non-inducing) or quinate (0.1%, w/v) and glycerol (0.2%, v/v) (inducing condition) and incubated at 37 °C with vigorous aeration for another 5 h. The mycelium was harvested by filtration and cell-free extracts were produced. Biosynthetic (type-I) 3-dehydroquinase activities were determined by assaying total 3-dehydroquinase activity and subtracting the heat-stable portion (stable after 10 min at 70 °C) which represents the catabolic (type-II) dehydroquinase activity. Specific activities are shown for shikimate dehydrogenase and biosynthetic (type-I) 3-dehydroquinase, in the wild-type strain R153, a putative gene-conversion transformant 1333, six potential overexpressors 1313–1351, and the positive control AROM overproducer QAT15 (Lamb *et al.* 1992b), for non-inducing conditions. The values in parentheses are the relative specific activities in comparison with the activity in the wild-type strain R153 under non-inducing conditions.

Strain	Specific activity of biosynthetic type-I 3-dehydroquinase and shikimate dehydrogenase in AROM-overproducing strains (μmol of product/mg of protein)			
	Non-induced (0.2% glucose)		Induced (0.1% quinate + 0.2% glycerol)	
	Shikimate dehydrogenase	3-Dehydroquinase	Shikimate dehydrogenase	3-Dehydroquinase
R153	0.011 (1.0)	0.0041 (1.0)	0.010 (0.9)	0.011 (2.7)
QAT15	0.012 (1.1)	0.0065 (1.6)	0.185 (17)	0.101 (24)
1313	0.055 (4.9)	0.0070 (1.7)	0.203 (18)	0.0450 (11)
1314	0.047 (4.2)	0.018 (4.3)	1.39 (124)	0.464 (113)
1328	0.025 (2.2)	0.009 (2.2)	0.335 (30)	0.078 (19)
1329	0.023 (2.0)	0.0100 (2.4)	0.387 (35)	0.120 (29)
1333	0.020 (1.8)	0.0092 (2.2)	0.015 (1.3)	nd
1348	0.030 (2.7)	0.0131 (3.2)	0.679 (61)	0.173 (42)
1351	0.024 (2.2)	0.0101 (2.5)	0.581 (52)	0.114 (28)

1179) which was unable to grow in minimal medium without supplementation with aromatic amino acids, and could not use quinate as carbon source, as it had no functional quinate dehydrogenase activity. This strain was therefore unable to catabolize quinate via quinate dehydrogenase, thereby potentially allowing quinate to act as a gratuitous inducer allowing greater levels of transcription of heterologous genes placed under the control of the *qutE* truncated promoter. As the *aromA* gene had proved refractory to all attempts at stable overexpression in *E. coli* driven by the *trc* promoter, we attempted elevated stable overexpression of the *aromA* gene in the double-mutant strain 1179.

Protoplasts from strain 1179 were transformed with plasmid pNUFC2 by selection for growth in minimal medium with glucose as carbon source. This selection regime was predicted to subject the *qutE* promoter in pNUFC2 to carbon catabolite repression, giving a selection pressure for multicopy transformants with the potential for quinate-inducible elevated overexpression. Some 39 transformants were selected at random, purified by streaking for single spores, and screened in a series of growth tests. The transformants were picked on to minimal medium containing glucose, glycerol, or glycerol plus quinate, and compared in growth response with controls R153 and QAT15. Strain R153 is wild-type with respect to the *aromA* gene, and strain QAT15 has the *aromA* gene under the control of the truncated *qutE* promoter but in a genetic background wild-type for quinate-utilization-pathway genes (Lamb *et al.*, 1991). Strain R153 is therefore a negative control and QAT15 is positive control. On the basis of these growth tests, the transformants fell into two groups: (1) those that grow as well as the R153 strain on all media (27 out of 39) and (2) those that had a significantly decreased growth rate on glycerol/quininate medium (12 out of 39). We predicted that the largest class were likely to be gene-conversion events and that the smaller group were potential overexpressors, as the shikimate dehydrogenase activity of the overproduced AROM protein may catabolize quinate to DHQ,

thereby allowing increased flux into the shikimate pathway, causing the toxicity effect reported by Lamb *et al.* (1991).

Six of the potential overexpressors, one potential gene convertant, QAT15 and R153 were grown in glucose minimal medium, subsequently shifted to quinate-induction medium for 5 h, and cell-free extracts were assayed for the AROM protein 3-dehydroquinase and shikimate dehydrogenase enzyme activities, which are shown in Table 5. Inspection of Table 5 shows that (a) quinate-inducible overproduction AROM protein was observed in six transformants and the QAT15 positive control and (b) the overproduction of AROM protein varied from 14- to approx. 120-fold and (c) the impaired growth on glycerol/quininate minimal medium is correlated with elevated AROM protein production.

Samples of the cell-free extracts were subjected to SDS/PAGE, and analysis of the Coomassie-Blue-stained gel showed that all the AROM-overproducing strains had a quinate-inducible protein of appropriate molecular mass (175 kDa). Transformant strain 1314 is currently used as a source of mycelium for the bulk purification of the *A. nidulans* AROM protein.

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