Genetic control of the protocatechuic acid pathway in *Aspergillus nidulans*

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(Received 18 November 1991; revised 13 January 1992; accepted 17 January 1992)

The genetic analysis of 16 recessive *pca* mutants in *Aspergillus nidulans* deficient in the metabolism of protocatechuic acid (PCA) has revealed seven functional genes. The seven gene loci are distributed over three chromosomes: *pcaA*, *pcaC* and *pcaD* on linkage group II; *pcaE*, *pcaF* and *pcaG* on group V, and *pcaB* on group VIII, where it shows linkage [recombination frequency (RF) = 6.9%] to the *gut* gene cluster controlling the degradation of quinic acid to PCA. Only two of the *pca* gene loci are closely linked: *pcaE* and *pcaG* (RF = 0.8%). The properties of the *gut* and *pca* mutants clearly demonstrate the separate identity and regulation of the converging pathways from quinate or benzoate to PCA, which in turn is oxidatively degraded through β -ketoadipate to TCA intermediates. Similarly, the mutants are not affected in the metabolism of salicylate to catechol and its oxidation to β -ketoadipate, although two genes (*pcaA* and *pcaF*) are required for the further metabolism of β -ketoadipate. Catechol dioxygenase is induced by growth in the presence of salicylate, and PCA dioxygenase by benzoate or quinate. Three groups of *pca* mutants (*pcaB*, *pcaD* and *pcaE*) are deficient in the induction of PCA oxygenase and accumulate PCA when grown in the presence of quinate or benzoate. All three *pcaE* mutants and the single *pcaA* strain totally lack PCA oxygenase activity, while a single *pcaB* mutant strain has properties tentatively suggesting a positive role in the induction of the PCA pathway.

Introduction

A wide variety of soil microorganisms are able to catabolize aromatic compounds (Dagley, 1971), which arise largely from the extracellular degradation of lignins (Cain, 1980). The many forms of substituted aromatic ring are transformed to metabolic intermediates converging upon central pathways in which the aromatic nucleus is cleaved oxidatively by either 'meta'- or 'ortho'-fission in bacteria and by 'ortho'-fission in fungi yielding, respectively, either γ - or β -ketoadipates which are further degraded to intermediates that enter the tricarboxylic acid cycle.

The two branches of the β -ketoadipate pathway are controlled by separate operons (Stanier & Ornston, 1973), and operons controlling such central pathways are often themselves clustered on the bacterial chromosome in 'supra-operons' (Wheelis, 1975). In contrast, enzymes of the peripheral pathways for aromatic transformations are usually encoded by plasmid borne genes (Holloway & Morgan, 1986). The isolation of gene clusters for *cat*, *ben* (benzoate) and *pca* metabolism from *Acinetobacter*

calcoaceticus by their expression in *Pseudomonas putida* (Shanley *et al.*, 1986) or *Escherichia coli* (Neidle *et al.*, 1989; Doten *et al.*, 1987) has enabled molecular analysis of the regulatory mechanisms. The strong likelihood of a quinate operon has not been investigated apart from the report of a quinate non-utilizing mutant strain *qui-1001* of *Pseudomonas putida* (Wheelis & Stanier, 1970).

An extensive survey by Cain *et al.* (1968) of the metabolism of aromatic acids in fungi confirmed the separate identities of the catechol and protocatechuate pathways, and demonstrated that fungi metabolize benzoate to *p*-hydroxybenzoate and then protocatechuate rather than to catechol, as is the case in bacteria. Moreover, fungi produced a β -carboxy muconolactone prior to β -ketoadipate rather than a γ -derivative. Generally, fungi show tight regulation of dioxygenase induction, and subsequent enzymes in the pathway are less rigorously controlled than in bacteria other than certain *Rhizobiaceae* (Parke & Ornston, 1986). The same routes for aromatic degradation by the protocatechuate pathway have been demonstrated in the soil yeasts *Rhodotorula mucilaginosa* (Cook & Cain, 1974) and *Trichosporon cutaneum* (Powlowski *et al.*, 1985).

It is striking that no genetic studies have been reported on aromatic catabolism in fungi apart from the initial steps in the degradation of quinate to protocatechuate, in

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

which the enzymes are highly inducible and controlled by a cluster of closely linked genes (Giles *et al.*, 1985). Quinate is a good carbon source for growth of *Aspergillus nidulans* and extensive genetic and molecular analysis of the *qut* gene cluster has been undertaken (Grant *et al.*, 1988; Lamb *et al.*, 1990). In addition the control of the aromatic biosynthetic pathway has been investigated to identify molecular mechanisms regulating gene expression to produce physiological integration between potentially competing metabolic pathways (Hawkins & Roberts, 1989; Lamb *et al.*, 1991). Isolation and analysis of the complex gene *aromA* from *A. nidulans* has shown an uninterrupted open reading frame encoding a single pentafunctional polypeptide catalysing steps 2 to 6 in the common pathway for aromatic biosynthesis (Charles *et al.*, 1986), and which has probably arisen by multiple gene fusions in divergent evolution from a common ancestor (Hawkins, 1987). The question of whether the genes controlling the β -ketoadipate pathway in fungi are closely linked has not been previously addressed. A gene, *bguA*, affecting the utilization of both benzoic and quinic acids has been located to linkage group II in *A. nidulans* (Hynes & Kelly, 1981).

A number of *pca* mutants deficient in the further metabolism of protocatechuate were recovered in the course of the isolation and analysis of quinate non-utilising mutants in *A. nidulans* (Grant *et al.*, 1988), and the analysis of 16 *pca* mutants identifying seven functional genes in the β -ketoadipate pathway is reported in this communication.

Methods

Aspergillus nidulans strains. The wild-type and mutant strains, culture conditions and media were all as previously described, and conventional methods were followed in crossing strains and in the synthesis of heterozygous diploids (Grant *et al.*, 1988). The *pca* genes were allocated to linkage groups by mitotic haploidization (Pontecorvo & Kafer, 1958) using a master strain MSE (*yA2*; *wA3*; *galA1*; *pyroA4*; *facA303*; *sB3*; *nicB8*; *riboB2*; McCully & Forbes, 1965) to synthesize the heterozygous diploid strains and benomyl ($0.7 \mu\text{g ml}^{-1}$) to induce mitotic haploidization (Hastie, 1970). Carbon sources were supplied at the final concentrations 20 mM-glucose, 40 mM-glycerol and 100 mM-acetate (at pH 6.5) from sterile stock solutions in distilled water autoclaved at 10 lbs (about 70 kPa) pressure for 10 min. Aromatic acids were supplied at final concentrations of 10 mM from stock solutions brought to pH 6.5–7.0 and sterilized by filtration except quinate which was autoclaved as above.

The Rothera test. This reaction tests for the accumulation of keto acids and was based upon the method of Norris & Ribbons (1971). Mycelium grown for 14–16 h in liquid minimal medium with glucose as carbon source was harvested by filtration under sterile conditions and briefly rinsed with glucose-free medium. Samples of mycelium were then resuspended in the same growth medium containing either no carbon source (negative control) or 10 mM aromatic acid, and incubated for 6 h before they were recovered on the filter. Approximately 0.1 g fresh weight mycelium was resuspended in 2 ml 0.02 M-Tris/HCl buffer pH 8.0 in a glass tube and 0.5 ml toluene was added.

The suspension was agitated periodically during a 1 h incubation at 30 °C before carrying out the Rothera reaction.

Approximately 1 g of $(\text{NH}_4)_2\text{SO}_4$ crystals were added to each tube, followed by 1 drop (about 0.05 ml) of freshly prepared 1% (w/v) sodium nitroprusside and then 0.5 ml 0.880 sp. gr. ammonia. The immediate development of a deep violet colouration upon mixing indicates the presence of keto acids. Small amounts of β -ketoadipate were added in place of mycelium as a positive control.

Enzyme assays

Mycelium was grown in glucose liquid culture and resuspended in liquid growth medium with a single aromatic substrate (0.01 M) and incubated for a further 12–16 h. The mycelium was then recovered on the filter, rinsed with distilled water and extraction buffer (0.1 M-potassium phosphate, pH 7.2, 10 mM-EDTA, 1 mM-DTT, 1 mM-PMSF). Mycelium was stored at -20°C as required and cell-free extracts prepared by grinding to a fine powder under liquid nitrogen in a cold mortar and pestle and subsequent extraction with buffer (Grant *et al.*, 1988). Protein concentrations were estimated using the Folin reagent and bovine serum albumin standards. All enzyme assays were done in a SP1800 recording spectrophotometer at 37 °C following the methods described in Norris & Ribbons (1971). Units of enzyme specific activity are $\Delta A \text{ min}^{-1} (\text{mg protein})^{-1}$.

PCA 3,4-dioxygenase (EC 1.13.11.3). The oxidation of PCA was followed as the decrease in A_{290} in a 1.0 ml reaction mixture containing 0.1 M-potassium phosphate buffer pH 7.0 and 0.01 M-PCA using 25–50 μl of cell-free extract.

Catechol 1,2-dioxygenase (EC 1.13.11.1) was assayed as the formation of *cis-cis*-muconate which absorbs strongly at 260 nm. The final reaction mixture was 3.0 ml containing 2.0 ml 0.1 M-potassium phosphate buffer pH 7.0; 0.4 ml 0.01 M-EDTA and 25–50 μl cell free extract. The volume was brought to 2.7 ml with distilled water and 0.3 ml water (reference cell) or 0.3 ml 0.01 M-catechol (sample cell) added to start the reaction.

Results and Discussion

Genetic analysis of *pca* mutants

Sixteen *pca* mutants identified in the isolation of *qut* mutants (Grant *et al.*, 1988) were analysed. These strains grow normally with glucose or acetate as carbon source but fail to grow on quinic acid (QA) medium. They are distinct from the *qut* mutants since they retain overall activity of the QUT pathway producing protocatechuic acid (PCA) from QA following growth upon glycerol in the presence of QA. All of the mutants proved recessive to the wild-type in heterozygous diploid strains and complementation analysis in diploids showed that the mutants fell into seven functional groups (designated *pcaA* to *pcaG*), several of which were represented by single mutant alleles (*pcaA*, 52, 98, 203, 343; *pcaB*, 164; *pcaC*, 180, 212, 226; *pcaD*, 198; *pcaE*, 120, 200, 253, 336; *pcaF*, 208; *pcaG*, 169, 244). Different mutants within each group exhibited identical phenotypic properties and a single representative strain was generally chosen for further analysis. Test crosses between the *pca* mutants and wild-type showed that in each case the mutant phenotype segregated as a single gene mutation.

Table 1. Allocations of *pca* mutations to linkage groups by mitotic haploidization

Linked markers assort into haploid strains from the heterozygous diploids only in the two parental configurations, and the classes essentially of this pattern are shown in **bold**. Markers on different chromosome pairs assort into four classes.

Linkage group	Strain of origin*	Known markers	Number of haploids†											
			<i>pcaA</i>	<i>pcaA</i> ⁺	<i>pcaB</i>	<i>pcaB</i> ⁺	<i>pcaC</i>	<i>pcaC</i> ⁺	<i>pcaD</i>	<i>pcaD</i> ⁺	<i>pcaE</i>	<i>pcaE</i> ⁺	<i>pcaF</i>	<i>pcaF</i> ⁺
I	MSE	<i>yA2</i>	0	21	4	8	0	29	0	12	14	18	11	11
	R21 <i>pca</i>	<i>pabaA1 yA2</i>	0	22	7	6	0	20	0	26	13	10	9	8
II‡	MSE	<i>wA3</i>	0	43	11	14	0	40	0	38	27	28	20	19
	R21 <i>pca</i>		0	0	0	0	0	0	0	0	0	0	0	0
III	MSE	<i>galA1</i>	0	22	5	9	0	18	0	15	8	12	6	11
	R21 <i>pca</i>		0	21	6	5	0	22	0	23	19	16	14	8
IV	MSE	<i>pyroA4</i>	0	18	5	9	0	19	0	19	11	12	11	12
	R21 <i>pca</i>		0	25	6	5	0	21	0	19	16	16	9	7
V	MSE	<i>facA</i>	0	25	6	5	0	21	0	22	1	24	0	16
	R21 <i>pca</i>		0	18	5	9	0	19	0	16	26	4	20	3
VI	MSE	<i>sB3</i>	0	23	6	7	0	26	0	20	13	15	10	14
	R21 <i>pca</i>		0	20	5	7	0	14	0	18	14	13	10	5
VII	MSE	<i>nicB8</i>	0	22	5	5	0	21	0	16	12	16	11	11
	R21 <i>pca</i>		0	21	6	9	0	19	0	22	15	12	9	8
VIII	MSE	<i>ribo2</i>	0	21	0	14	0	20	0	26	13	13	13	10
	R21 <i>pca</i>		0	22	11	0	0	20	0	12	14	15	7	9

* The haploid component strains of the heterozygous diploids were the master strain (MSE), the genotype of which is given in the text and *pabaA1 yA2*; *pca* (R21 *pca*). The mutant alleles tested were *pcaA52*, *pcaB164*, *pcaC180*, *pcaD198*, *pcaE200* and *pcaF208*.

† The heterozygous diploid strains produced yellow conidiospores as the result of homozygosity for the *yA2* marker. Therefore only white-spored strains could be recovered upon haploidization and these were classified as haploid upon the segregation and expression of at least two of the nutritional markers. The segregation of linkage group I was followed by the marker *pabaA1*.

‡ In three cases (*pcaA*, *pcaC*, *pcaD*) only non-mutant *pca*⁺ haploid segregants were recovered. Allocation of these gene loci to chromosome II is provisional based upon recovery of one class of segregants (*wA3 pca*⁺), since the other would have yellow spores (*wA⁺ pca*⁺) and thus not be identifiable. The random assortment of all seven other markers in these segregants strongly supports the location of these *pca* genes in linkage group II.

When the *pca* mutants were inter-crossed only one pair of genes was found closely linked *pcaE* and *pcaG* (RF = 0.8% – two recombinant QA⁺ progeny recovered in 500 ascospores plated). The *pca* mutants were also tested for linkage to the *qut* gene cluster, and one gene was found to map proximal to the cluster on chromosome VIII in the order *pcaB164*–6.9% – *qutD312*–4.4% – *qutR16* with the recombination fractions shown.

Heterozygous diploid strains between representative *pca* mutant strains and a 'master strain' MSE (McCully & Forbes, 1965) were synthesized to allocate the *pca* genes to linkage groups by mitotic haploidization (Table 1). Three genes were provisionally located to linkage group II (*pcaA pcaC pcaD*) and one of these most probably represents the *bguA* mutant of Hynes & Kelly (1981), which was not available for analysis. Three genes were located to linkage groups V (*pcaE* and *pcaF*; together with *pcaG* by close linkage to *pcaE*), while the location of *pcaB* linked to the *qut* gene cluster on chromosome VIII was confirmed.

Thus the seven *pca* gene loci analysed fall to three of the eight linkage groups in *A. nidulans*, but most strikingly only two show close linkage, *pcaE* and *pcaG* (RF 0.8%), and only one gene, *pcaB*, is linked to the *qut*

gene cluster. This distribution of the seven *pca* genes stands in marked contrast to the tight linkage of all seven genes in the *qut* gene cluster.

Further characterization of the *pca* mutants

The *pca* mutants show reduced growth on PCA as expected and their very poor growth on benzoate or *p*-hydroxybenzoate suggests the metabolism of these compounds by the PCA pathway (Fig. 1). In addition, certain mutants produce an immediate reaction in the 'spot-test' for PCA (Grant *et al.*, 1988) due to the prior accumulation of PCA during growth of mycelium in the presence of QA, indicating a metabolic lesion in enzyme(s) early in the PCA pathway (Table 2). Normal growth of the *qut* mutants on benzoate and PCA indicates separate genetic control of the QUT pathway, also reflected by normal growth of the QA non-inducible activator mutant (*qutA4*) and a mutant (*qutD312*) lacking quinate permease. The ability of all of the *qut* mutant strains and most of the *pca* mutants to grow normally on salicylate medium strongly suggests separate pathways for catechol and PCA metabolism in *A. nidulans*. Poor growth of strains *pcaA52*, *pcaB164* and

Table 2. Utilization of aromatic acids for growth by strains of *A. nidulans*

The conversion of quinic acid (QA) to protocatechuic acid (PCA) was determined by a 'colorimetric spot-test' for PCA (Grant *et al.*, 1988) applied to mycelium grown on glycerol (20 mM) agar plates in the presence of QA (0.5%, w/v). The formation of PCA in the reaction system upon overnight incubation was recorded + and an immediate reaction + (imm.). All aromatic acids were tested at 10 mM in minimal agar medium at pH 6.5, and growth responses scored after 48 h incubation on a scale 0 (no growth) to 5. In this scale growth of the wild-type on glucose would be scored 5 and on acetate 4 to reflect the different growth yields. A series of *qut* mutants were included for comparison and mutants *qutD312*, *qutB42*, *qutE208* and *qutC113* gave the same results as the non-inducible mutant *qutA4*. The mutants are deficient in enzymes for steps 1 (*qutB*), 2 (*qutE*) or 3 (*qutC*) in the metabolism of QA to PCA, or in a QA permease (*qutD*). The mutant *qut16* is a recessive constitutive strain defective in a repressor protein (Grant *et al.*, 1988). All strains were in the R153 (*wA3*; *pyroA4*) genetic background.

Strains	Conversion QA→PCA	Carbon source for growth				
		Quinic acid	Benzoic acid	<i>p</i> -Hydroxy benzoic acid	Salicylic acid	Protocatechuic acid
Wild-type (R153)	+	4	4	4	2	3
<i>pcaA52</i>	+	0	0	1	1	1
<i>pcaB164</i>	+	0	0	0	1	0
<i>pcaC180</i>	+	0	0	1	2	1
<i>pcaD198</i>	+ (imm.)	1	1	1	2	1
<i>pcaE200</i>	+ (imm.)	1	0	1	2	1
<i>pcaF208</i>	+	0	0	1	1	1
<i>pcaG244</i>	+	0	0	0	2	0
<i>qutA4</i>	—	0	4	4	2	3
<i>qutR16</i>	+	4	4	4	2	3

Table 3. Accumulation and production of keto-acid intermediates in strains of *A. nidulans* grown in the presence of different aromatic acids

The strains were grown in minimal medium in liquid culture for 14–16 h with glucose as carbon source. Mycelium was harvested on a filter, washed briefly with the same medium (at 37 °C) without glucose and resuspended in further minimal growth medium with the aromatic acids indicated at 10 mM and at pH 6.5. Mycelium was collected after a further 6 h incubation and the Rothera test applied to samples as described in Methods. The Rothera reactions were done without added substrate (none) or with PCA or catechol at 10 mM. The *qutA4* strain is described in the legend to Table 2. Strong accumulation or production of keto acids is shown +, weak (+) and no accumulation —.

Strain	Substrate in Rothera test	Aromatic acid in growth medium					
		Quinic acid		Benzoic acid		Salicylic acid	
		None	PCA	None	PCA	None	Catechol
Wild-type (R153)		—	+	—	+	—	+
<i>pcaA52</i>		+	+	+	+	+	+
<i>pcaB164</i>		(+)	(+)	—	—	—	(+)
<i>pcaC180</i>		+	+	+	+	—	+
<i>pcaD198</i>		—	—	—	—	—	+
<i>pcaE200</i>		—	—	—	—	—	+
<i>pcaF208</i>		+	+	+	+	+	+
<i>pcaG244</i>		+	+	+	+	—	+
<i>qutA4</i>		—	—	—	+	—	+

Fig. 1 (facing page). β -Ketoadipic acid pathway in *A. nidulans*. Reactions in the metabolism of benzoic, quinic and salicylic acids to β -ketoadipic acid are indicated by arrows. Intermediates numbered are 1, β -carboxy-*cis,cis*-muconic acid; 2, β -carboxymuconolactone; 3 (hypothetical), γ -carboxy- β -ketoadipate-enol-lactone; 4 (hypothetical), γ -carboxy- β -ketoadipic acid; 5, *cis,cis*-muconic acid; 6, (+)-muconolactone. Aromatic ring cleavage by PCA 3,4-dioxygenase and by catechol 1,2-dioxygenase are shown by O₂ uptake. Genes controlling enzymes in the PCA pathway are shown. The genes *pcaD* and *pcaE* control PCA dioxygenase and the provisional allocation of genes controlling other enzymes is indicated. The mutant strain *pcaB* has characteristics indicating that this gene has a positive role in the induction of the enzymes of the pathway and is discussed in the text.

pcaF208 on benzoate, *p*-hydroxybenzoate and salicylate indicated that these mutants are deficient in enzymes below the common intermediate β -ketoadipate produced by convergence of the catechol and PCA pathways. Use of the Rothera test for the presence of keto acids supports these suggestions (Table 3). Most of the *pca* mutants accumulate keto acids following incubation in the presence of quinate or benzoate, suggesting metabolic lesions following aromatic ring cleavage. Moreover, the *pcaD* and *pcaE* mutants do not accumulate keto acids or produce them from PCA, suggesting deficiency in ring cleavage, and consistent with the observed accumulation of PCA in these strains when incubated in the presence of QA. All of the *pca* mutants produce keto acids from catechol following growth in the presence of salicylate, again demonstrating the separate identity of the catechol pathway.

Interestingly, strains *pcaA52* and *pcaF208* accumulate keto acids during growth in the presence of salicylate, indicating lesion(s) below β -ketoadipate. Strain *pcaB164* shows unusual responses and is discussed below. Absence of keto acid accumulation in the QA non-inducible *qutA* mutant and production of keto acids from PCA following induction by benzoate or from catechol by salicylate, demonstrates that the *QUT* activator protein specifically regulates the QUT pathway and the *qut* gene cluster and is not required for induction of the PCA or catechol pathways.

Induction of PCA and catechol dioxygenase activities

Neither of the two oxygenases was detectable in glucose-grown wild-type mycelium. PCA oxygenase was induced following growth on benzoic acid (0.26 units specific activity) or quinic acid (0.19 units) while catechol oxygenase was induced by growth on salicylic acid (0.25 units). (See Methods for details of these experiments.) Although a good carbon source for growth, *p*-hydroxybenzoate was a poor inducer of PCA oxygenase (0.08 units) as was PCA supplied exogenously (0.06 units). Vanillate or gentisate induced no detectable PCA oxygenase activity and were also poor substrates for growth. Low levels of apparent catechol oxygenase activity found after growth with aromatic acids other than salicylate may represent very low level induction or residual activity of PCA oxygenase for catechol (data not shown). PCA oxygenase was induced by benzoate in a QA non-inducible *qutA* mutant (Table 4), again demonstrating pathway-specific control and strongly suggesting that PCA is the intracellular inducer of PCA oxygenase and perhaps the other enzymes of the PCA pathway.

Three groups of *pca* mutants were found to be deficient in PCA oxygenase activity, *pcaB*, *pcaD* and *pcaE* (Table 4). Mutants in the *pcaE* gene (three alleles) and the single

Table 4. PCA oxygenase activities in *pca* mutants of *A. nidulans*

Mycelium of wild-type strain R153 and the mutant strains was grown in liquid culture with glucose as carbon source and subsequently incubated for 12 h in growth medium with the substrates shown (all at 10 mM and pH 6.5) as described in the legend to Table 2. Cell-free extracts were prepared and assayed as described in Methods. Specific enzyme activities are $\Delta A \text{ min}^{-1} (\text{mg protein})^{-1}$. The wavelengths were 290 nm for PCA oxygenase and 260 nm for catechol oxygenase. The specific activity values are means of three replicate assays of each extract between which good concurrence was observed. Assays done on different occasions are shown separately and grouped into the upper and lower panels of the Table. The relative enzyme activities observed in each experiment in comparison to the wild-type control (1.0) are shown in parentheses. ND, No enzyme activity detected.

Strain	PCA oxygenase			Catechol oxygenase
	Induced by quinic acid		Induced by benzoic acid	Induced by salicylic acid
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Wild-type	0.121 (1.00)	0.143 (1.00)	0.067 (1.00)	0.19 (1.00)
<i>pcaA52</i>	0.034 (0.28)		0.017 (0.25)	0.10 (0.53)
<i>pcaB164</i>	0.002 (0.02)	0.005 (0.03)	ND	0.17 (0.89)
<i>pcaD198</i>	ND		ND	
<i>pcaE200</i>	ND		ND	
<i>pcaE120</i>	ND			
<i>pcaE336</i>	ND			
<i>qutA4</i>		ND	0.053 (0.79)	

Strain	PCA oxygenase	Catechol oxygenase
	Induced by quinic acid	Induced by salicylic acid
	Experiment 5	Experiment 6
Wild-type	0.096 (1.00)	0.18 (1.00)
<i>pcaC180</i>	0.048 (0.50)	
<i>pcaF208</i>	0.054 (0.57)	0.21 (1.17)
<i>pcaG244</i>	0.024 (0.25)	

mutant *pcaD198* had essentially no PCA oxygenase activities, very similar properties in the accumulation of PCA from QA (Table 2) and inability to form α -keto acids from PCA (Table 3). These strains are therefore deficient in ring cleavage due to lack of PCA oxygenase but the precise roles of the *pcaD* and *pcaE* genes in encoding the polypeptides constituting the holoenzyme remains to be established.

The single mutant allele *pcaB164* produces very low levels of PCA oxygenase activity (Table 4) and shows reduced accumulation of α -keto acids after growth in the presence of QA (Table 3). The *pcaB164* strain induces the three *QUT* specific enzyme activities at levels close to a wild-type strain (data not shown) and is therefore not deficient in the induction of the QUT pathway. It remains an attractive hypothesis that the *pcaB* gene locus has a positive role in the specific induction of the PCA pathway, and the significance of linkage of the *pcaB* gene to the *qut* gene cluster remains to be explored.

This work was in part supported by an award to Kuswandi from the World Bank Indonesia 2 Project.

References

- CAIN, R. B. (1980). The uptake and catabolism of lignin-related aromatic compounds and their regulation in microorganisms. In *Lignin Degradation: Microbiology, Chemistry and Potential Applications*, vol. 1, pp. 21–60. Edited by T. Kent Kirk, T. Higuchi & H. Chang. Boca Raton, Fla.: CRC Press.
- CAIN, R. B., BILTON, R. F. & DARRAH, J. A. (1968). The metabolism of aromatic acids by microorganisms. *Biochemical Journal* **108**, 797–828.
- CHARLES, I. G., KEYTE, J. W., BRAMMAR, W. J., SMITH, M. & HAWKINS, A. R. (1986). The isolation and nucleotide sequence of the complex *arom* locus of *Aspergillus nidulans*. *Nucleic Acid Research* **14**, 2201–2213.
- COOK, K. A. & CAIN, R. B. (1974). Regulation of aromatic metabolism in the fungi. Metabolic control of 3-oxoadipate pathway in the yeast *Rhodotorula mucilaginosa*. *Journal of General Microbiology* **85**, 37–50.
- DAGLEY, S. (1971). Catabolism of aromatic compounds by microorganisms. *Advances in Microbial Physiology* **6**, 1–42.
- DOTEN, R. C., NGAI, K. L., MITCHELL, D. J. & ORNSTON, L. N. (1987). Cloning and genetic organization of the *pca* gene cluster from *Acinetobacter calcoaceticus*. *Journal of Bacteriology* **169**, 3168–3174.
- GILES, N. H., CASE, M. E., BAUM, J., GEIVER, R., HUIET, L., PATEL, V. & TYLER, B. (1985). Gene organization and regulation in the *qa* (quinic acid) gene cluster of *Neurospora crassa*. *Microbiological Reviews* **49**, 338–358.
- GRANT, S., ROBERTS, C. F., LAMB, H., STOUT, M. & HAWKINS, A. R. (1988). Genetic regulation of the quinic acid utilization (*QUT*) gene cluster in *Aspergillus nidulans*. *Journal of General Microbiology* **134**, 347–358.
- HASTIE, A. C. (1970). Benlate induced mitotic instability of *Aspergillus* diploids. *Nature, London* **226**, 771.
- HAWKINS, A. R. (1987). The complex *AROM* locus of *Aspergillus nidulans*: evidence for multiple gene fusions and convergent evolution. *Current Genetics* **11**, 491–498.
- HAWKINS, A. R. & ROBERTS, C. F. (1989). Molecular interactions between the quinic acid catabolic and shikimate pathways in *Aspergillus nidulans*. In *Molecular Biology of Filamentous Fungi*, vol. 6, pp. 85–100. Edited by H. Nevalainen & M. Penttilä. Helsinki: Foundation for Biotechnical and Industrial Research.
- HOLLOWAY, B. W. & MORGAN, A. F. (1986). Genome organization in *Pseudomonas*. *Annual Review of Microbiology* **40**, 79–105.
- HYNES, M. J. & KELLY, J. M. (1981). Threonine, quinate and glucuronic acid utilization mutants of *Aspergillus nidulans*. *Aspergillus News Letter* **15**, 18–21.
- LAMB, H. K., HAWKINS, A. R., SMITH, M., HARVEY, I. J., BROWN, J., TURNER, G. & ROBERTS, C. F. (1990). Spatial and biological organization of the complete quinic acid utilization gene cluster in *Aspergillus nidulans*. *Molecular and General Genetics* **223**, 17–23.
- LAMB, H. K., BAGSHAW, C. R. & HAWKINS, A. R. (1991). *In vivo* overproduction of the pentafunctional AROM polypeptide in *Aspergillus nidulans* affects the metabolic flux in the quinate pathway. *Molecular and General Genetics* **227**, 187–196.
- MCCULLY, K. S. & FORBES, E. (1965). The use of *p*-fluorophenylalanine with 'master strains' of *Aspergillus nidulans* for assigning genes for linkage groups. *Genetical Research* **6**, 352–359.
- NEIDLE, E. L., HARTNETT, C. & ORNSTON, L. N. (1989). Characterizations of *Acinetobacter calcoaceticus* CatM, a repressor gene homologous in sequence to transcriptional activator genes. *Journal of Bacteriology* **171**, 5410–5421.
- NORRIS, J. R. & RIBBONS, D. W. (1971). *Methods in Microbiology*. London: Academic Press.
- PARKE, D. & ORNSTON, L. N. (1986). Enzymes of the β -ketoadipate pathway are inducible in *Rhizobium* and *Agrobacterium* spp and constitutive in *Bradyrhizobium* spp. *Journal of Bacteriology* **165**, 288–292.
- PONTECORVO, G. & KAUFER, E. (1958). Genetic analysis based upon mitotic recombination. *Advances in Genetics* **9**, 71–104.
- POWLOWSKI, J. B., INGEBRAND, J. & DAGLEY, S. (1985). Enzymology of the β -ketoadipate pathway in *Trichosporon cutaneum*. *Journal of Bacteriology* **163**, 1136–1141.
- SHANLEY, M. S., NEIDLE, E. L., PARALES, R. E. & ORNSTON, L. N. (1986). Cloning and expression of *Acinetobacter calcoaceticus* catBCDE genes in *Pseudomonas putida* and *Escherichia coli*. *Journal of Bacteriology* **165**, 557–563.
- STANIER, R. Y. & ORNSTON, L. N. (1973). The β -ketoadipate pathway. *Advances in Microbial Physiology* **9**, 89–150.
- WHEELIS, M. L. (1975). The genetics of dissimilatory pathways in *Pseudomonas*. *Annual Review of Microbiology* **29**, 505–524.
- WHEELIS, M. L. & STANIER, R. Y. (1970). The genetic control of dissimilatory pathways in *Pseudomonas putida*. *Genetics* **66**, 245–266.