Disruption of *phaC*, an *Aspergillus nidulans* Gene Encoding a Novel Cytochrome P450 Monooxygenase Catalyzing Phenylacetate 2-Hydroxylation, Results in Penicillin Overproduction*

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José Manuel Mingot, Miguel Angel Peñalva‡, and José Manuel Fernández-Cañón§

From the Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas del Consejo Superior de Investigaciones Científicas, Vélezáquez 144, Madrid, 28006, Spain

*Aspergillus nidulans* utilizes phenylacetate as a carbon source via homogentisate, which is degraded to furmarate and acetooacetate.Mutual evidence strongly suggested that phenylacetate is converted to homogentisate through two sequential hydroxylating reactions in positions 2 and 5 of the aromatic ring. Using cDNA substra technique, we have characterized a gene, denoted *phaC*, whose transcription is strongly induced by phenylacetate and which putatively encodes a cytochrome P450 protein. A disrupted *phaC* strain does not grow on phenylacetate but grows on 2-hydroxy- or 2,5-dihydroxyphenylacetate. Microsomal extracts of the disrupted strain are deficient in the NADPH-dependent conversion of phenylacetate to 2-hydroxyphenylacetate. We conclude that PhacA catalyzes the ortho-hydroxylation of phenylacetate, the first step of *A. nidulans* phenylacetate catabolism. The involvement of a P450 enzyme in the ortho-hydroxylation of a monoaromatic compound has no precedent. In addition, PhacA shows substantial sequence divergence with known cytochromes P450 and defines a new family of these enzymes, suggesting that saprophytic fungi may represent a source of novel cytochromes P450.

Phenylacetate is a precursor for benzylpenicillin production. *phaC* disruption increases penicillin production 3-5-fold, indicating that catabolism competes with antibiotic biosynthesis for phenylacetate and strongly suggesting strategies for *Penicillium chrysogenum* strain improvement by reverse genetics.

Aerobic degradation of aromatic hydrocarbons by microbes involves the action of oxygenases (enzymes that incorporate one or two atoms from dioxygen into substrates) acting at two different levels in specific catabolic pathways (1, 2). First, oxygenase enzymes acting at the upstream segment of these pathways incorporate one (monooxygenases, aromatic ring hydroxylases) or two (aromatic ring dioxygenases) oxygen atoms into the aromatic substrate as hydroxyl groups, preparing the ring for a subsequent ring-opening step. In this second step, the dihydroxylated aromatic ring is opened by ring-cleavage dioxygenases.

Monooxygenases are a mechanistically diverse group of enzymes (1) including, for example, flavoproteins such as 

$$ \text{p-hydroxybenzoate hydroxylase} $$

(3), multicomponent enzymes such as *Pseudomonas mendocina* toluene 4-monoxygenase, in which one of the terminal dioxygenase polypeptides contains a binuclear iron cluster (4), or heme-containing cytochrome P450 systems. Monooxygenases of the cytochrome P450 superfamily (5, 6) are widely distributed among living organisms and catalyze a multiplicity of biosynthetic and catabolic reactions, usually with narrow substrate specificity, including the hydroxylation of a variety of lipophylic drugs.

In common with other saprophytic microbes, the genetically amenable, obligate aerobic fungus *Aspergillus nidulans* shows notable metabolic versatility. For example, it can use the aromatic hydrocarbon compound phenylacetate (PhAc) as sole carbon source. Despite the abundant information available on the catabolic pathways of other aromatic compounds, which have been extensively studied in bacteria, our understanding of PhAc degradation pathways is scarce. In *Pseudomonas putida* U, it is known that PhAc is degraded through phenylacetyl-CoA (7), although the ring cleavage steps remain uncharacterized. In *A. nidulans*, PhAc degradation proceeds through 2,5-dihydroxy-PhAc (homogentisate, see Fig. 1). The three structural genes mediating the conversion of homogentisate to Krebs cycle intermediates (i.e. the “lower” PhAc pathway) have been characterized (8–10) but the steps leading to homogentisate have not yet been reported. We describe here mutational and molecular analysis showing that *A. nidulans* PhAc catabolism proceeds via homogentisate through two sequential hydroxylating steps, of which the first is the 2-hydroxylation of the ring catalyzed by a novel cytochrome P450. Targeted inactivation of this gene results in penicillin overproduction.

**EXPERIMENTAL PROCEDURES**

**Fungal Strains, Media, and Growth Conditions—*A. nidulans* strains**

marked carriers in standard use (11). Standard media for *A. nidulans* (12) were used for strain maintenance, growth tests, and transformation. Complementation tests were carried out in standard diploids. A *bia1* strain was the source of cDNA, and a *bia1 methG1 argB2* strain was the recipient strain for *phaC* gene disruption. A *bia1* *methG1* strain was used as wild type control in experiments with the disrupted strains. Culture conditions inducing high levels of expression of the PhAc catabolic genes have been described (13) and were used to grow mycelia for protein extraction. PhAc and its monohydroxy and dihydroxy derivatives were used as sole carbon source at 10 mM (although homogentisate was occasionally used at 25–50 mM), and 10 mM ammonium chloride was used as sole nitrogen source.

1 The abbreviations used are: PhAc, phenylacetate; CYP, cytochrome P450; kbp, kilobase pair(s); HPLC, high performance liquid chromatography.
Novel Cytochrome P450 in Fungal Phenylacetate Catabolism

Isolation of PhAc Nonutilizing Mutants—2-, 3-, and 4-fluorophenylacetate at a 5 mM concentration were shown to prevent growth of A. nidulans in the presence of a derepressing carbon source, indicating that their catalabolism was toxic for the mold. Moreover, some residual growth was observed when plates were incubated for more than 3 days at 37 °C. Sectors of markedly more vigorous mycelia formed after prolonged incubation. Although the reason why catalabolism of these PhAc derivatives results in toxicity is not clear, preliminary tests showed that mutations preventing the toxicity of 2- and 3-fluorophenylacetate also prevented the catalabolism of PhAc. Therefore, conidiospores of a ya2 phacB100 strain were plated to obtain isolated colonies, which were transferred to minimal medium with 0.05% lactate (w/v) as a carbon source in the presence of 5 mM 2- or 3-fluorophenylacetate. Sectors with more vigorous growth were purified and tested for the utilization of different PhAc derivatives as sole carbon source. Two major classes were found, which were denoted class I and II (see “Results”).

*phacA* Gene Disruption—A pUC18-based plasmid denoted pPhAcArgB was constructed by standard techniques. This plasmid contains (starting from the lacZ promoter in PUC18) 0.94 kb of the *phacA* upstream region sequentially followed by its genomic coding region up to codon 297, a genomic, 3.2-kb fragment containing an argB′ allele, the genomic sequence of *phacA* corresponding to codons 393–518, and finally 1.2 kb of the *phacA* 3′-downstream region. This insert was cloned in the plasmid containing the ura4-52 marker and used for transformation (14). Transformed (arginine-independent) clones in which the resident *phacA* gene had been replaced by the transforming fragment were identified by Southern analysis. The mutated allele would encode a protein truncated at residue 297 and therefore would lack the predicted region involved in heme binding.

Characterization of *phacA* cDNA and Genomic Clones—*phacA* cDNA clones were obtained by differential screening of a cDNA library enriched in PhAc-induced transcripts, as described (8–10). Seven cDNA clones were obtained. The insert of one such clone was used to isolate genomic clones from a standard λEMBL4 library. Cross-hybridizing sequences were mapped to two contiguous BamHI fragments, 2.4 kb and 1.9 kb long (a BamHI site was shown to split the *phacA* open reading frame). Nucleotide sequencing and comparison of genomic and cDNA clones revealed the intron-exon organization of the gene and showed that all seven cDNA clones were incomplete at the N-terminal coding region, the longest of which (at the 5′-end) ended within predicted codon 6. cDNAs including the predicted initiation codon were obtained by direct polymerase chain reaction amplification of the cDNA library using an internal *phacA* primer and a 10-specific primers followed by a second polymerase chain reaction reaction primed with the above internal oligonucleotide and a second oligonucleotide ending for 15 min. Microsomal pellets were recovered after centrifugation at 100,000 × g for 1 h and resuspended in 100 mM potassium phosphate buffer, pH 7.0. These extracts contained 1–4 mg/ml protein. Enzyme activities of the microsomal extract were determined using standard procedures (15, 16) with minor modifications. NADPH-cytochrome *P450* reductase was assayed in 1-mL reactions at 25 °C with 0.1 mM phosphate buffer, pH 7.0, following the NADPH-dependent reduction of cytochrome *c* (0.05 mM initial concentration; ε280 = 21.1 M−1 cm−1) or ferricyanide (0.5 mM initial concentration; ε420 = 1.92) by the decrease of absorbance at 550 nm and 420 nm, respectively.

PhAc 2-hydroxylase was assayed by measuring the formation of 2-hydroxy-PhAc in a reaction that required PhAc (added at 1 mM) and NADPH (also at 1 mM) in the presence of a microsomal fraction. 2-Hydroxy-PhAc was chemically determined using diazotized 4-nitroaniline (17). Absorbance was read at 551 nm and converted to nmol of 2-hydroxy-PhAc using a reference plot. The range of absorbance at 550 nm and 420 nm, respectively.

**RESULTS**

The "Upper" Phenylacetate Degradation Pathway—We found that 2-, 3-, or 4-fluorophenylacetate prevents *A. nidulans* growth on 0.05% (w/v) lactose as carbon source. We therefore selected surviving mutants resulting in fluorophenylacetate resistance, assuming that they would prevent PhAc utilization and following Apirion (19), who used fluorocatechol resistance to select acetate nonutilizing mutants. Mutations preventing PhAc utilization (*phac*) were efficiently selected with 2-fluorophenylacetate. They were recessive in diploids, indicating that they represent loss-of-function mutations. They were classified in two major classes. Class I mutants did not grow on PhAc but grew on 2-hydroxy-PhAc or 2,5-dihydroxy-PhAc. By contrast, class II mutants did not grow on either PhAc or 2-hydroxy-PhAc but grew on 2,5-dihydroxy-PhAc. Class I or II mutations did not affect growth on acetate, Phe, Tyr, 3- or 4-hydroxy-PhAc, and 3,4-dihydroxy-PhAc, showing that they specifically prevented PhAc catalabolism. Mutations in class I complemented class II mutations. As 2,5-dihydroxy-PhAc is known to be an intermediate of PhAc catalabolism (8–10), these data are consistent with the pathway shown in Fig. 1 in which PhAc is converted into homogentisate through two hydroxylating reactions, prevented by class I and class II mutations, respectively.

**Molecular Cloning of phacA, a Gene Encoding a Novel Cytochrome P450**—We have previously used a differential screening procedure of a subtracted cDNA library to isolate cDNA clones representing transcripts induced by PhAc (8–10). This collection of cDNAs included clones for the three genes (*fahA, maI*, and *hmgA*) of the lower PhAc pathway (Fig. 1; Refs. 8–10). Among the remaining cDNA clones, those that did not represent these previously described genes were identified and classified by restriction enzyme mapping and/or partial cDNA...
DNA fragment carrying a mutated phacA transformed an disruption-deletion
volvement of PhacA in PhAc catabolism, we constructed a phacA
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The transcript was absent in cells transferred to glucose alone
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data bases revealed that all 20 entries showing the highest amino
zymes, and PhacA residues 431 to 439 contain the peptide motif
bream) P450 protein. Cytochromes P450 are heme-thiolate en-
2 David R. Nelson, personal communication.
FIG. 2. Northern analysis of phacA transcript levels. Cells were grown on minimal medium with 0.3% (w/v) glucose as sole carbon source for 16 h at 37 °C and transferred to media with the indicated carbon sources (glucose at 1% (w/v), all aromatic compounds, and glutamate at 10 mM and potassium acetate at 30 mM; −carbon indicates no carbon source added). These secondary cultures were incubated for a further 1 h at 37 °C. Mycelia were then harvested and used to isolate RNA (18). The probe was a 1.2 kb phacA cDNA clone (4FG4). Actin transcript was used as loading control.
sequencing. Seven overlapping cDNAs represented a novel PhAc-induced transcript whose gene was named phacA and which contained an open reading frame putatively encoding a 518-residue polypeptide (M, 58,495). DNA sequencing of genomic clones showed that the phacA-coding region is interrupted by three introns, 65, 56, and 53 nucleotides long. The nucleotide sequence of phacA and the amino acid sequence of its derived protein product have been deposited in the DDBJ/EMBL/GenBank data bases under accession number AJ132442. Blast searches against nonredundant Swissprot+Translation of EMBL nucleotide sequence data bases revealed that all 20 entries showing the highest amino
Identity levels were in the 25% range, with the highest identity (27.4% in a 465 residue overlap) shown by a Sparus aurata (gilthead sea bream) P450 protein. Cytochromes P450 are heme-thiolate en-
zymes, and PhacA residues 431 to 439 contain the peptide motif Gly-Xaa-Gly-Xaa-Xaa-Cys-Xaa-Gly (where Xaa indicates any amino acid), which is involved in heme binding in proteins of this class (6). We conclude that phacA encodes a cytochrome P450. The above levels of identity are below those required to place it in an already existing family (>40% identity required (5)). Therefore, PhacA defines a new family of these proteins. It has been denoted CYP504 using the current P450 nomenclature system.
phacA Is Strongly Induced by Phenylacetate—phacA transcript levels were analyzed by Northern analysis in cells grown in glucose and subsequently transferred to minimal media, each containing a different carbon source. This analysis (Fig. 2) showed that phacA transcription is strongly induced by PhAc and largely repressed by glucose (PhAc plus glucose, Fig. 2). The transcript was absent in cells transferred to glucose alone or to gluconeogenic substrates acetate or glutamate. 2-Hydroxy-PhAc was also a strong inducer (although less so than PhAc), and 3-hydroxy-PhAc was a weak inducer. 4-Hydroxy-PhAc, 2.5-, or 3,4-dihydroxy-PhAc did not induce phacA trans-
scription. Notably, Phe (but not Tyr) induced phacA transcription to some extent. These results support the contention that phacA is involved in PhAc catabolism.
The Phenotype of a Disruption Strain Is Consistent with phacA Encoding a PhAc 2-Hydroxylase—To confirm the in-
volvement of PhacA in PhAc catabolism, we constructed a disruption-deletion phacA mutation by reverse genetics. We transformed an argB2 arginine-requiring strain with a linear DNA fragment carrying a mutated phacA gene in which a 3.2-kbp fragment containing an argB+ allele replaced a 289-base pair NaeI-KpnI phacA genomic fragment including codons 298–392 (see Fig. 3). This mutant phacA gene would encode a PhacA protein truncated at residue 297. Two transformants (denoted ΔphacA #3 and #4) carrying the expected disruption-deletion mutation were purified and tested for growth on minimal medium with 0.05% (w/v) lactose, 10 mM PhAc, or 10 mM 2-hydroxy-PhAc as sole carbon source, as indicated. A wild type strain and a strain carrying a null (ΔhmgA) mutation in the homogen-
tosidase dioxygenase gene (see Fig. 1) were used as controls. Plates were incubated for 4 days at 37 °C before being photographed.

FIG. 3. Disruption of phacA. The phacA gene was replaced by a mutant version after transformation with a linear DNA fragment in which phacA codons 298–392 had been replaced by a 3.2-kbp DNA fragment containing the argB+ gene (see “Experimental Procedures”). This mutant allele encodes a PhacA protein truncated after residue 297. Two transformants (denoted ΔphacA #3 and #4) carrying the expected disruption-deletion mutation were purified and tested for growth on minimal medium with 0.05% (w/v) lactose, 10 mM PhAc, or 10 mM 2-hydroxy-PhAc as sole carbon source, as indicated. A wild type strain and a strain carrying a null (ΔhmgA) mutation in the homogen-
tosidase dioxygenase gene (see Fig. 1) were used as controls. Plates were incubated for 4 days at 37 °C before being photographed.

lactose PhAc 2-0Hl-PhAc

ΔhmgA

argB+

phacA

ΔphacA

#3

#4

wt

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PhAc and NADPH, we detected the formation of a monohydroxylated PhAc derivative using a chemical detection method ((Fig. 4A; see “Experimental Procedures”). Direct HPLC analysis of the reaction mixture showed that this compound was 2-hydroxy-PhAc and that its formation was absolutely dependent on the presence of both PhAc and NADPH (Fig. 4B). This showed that A. nidulans microsomes contain a phenylacetate ortho-hydroxylating activity. This activity was markedly and reproducibly reduced but not abolished by the \( \Delta \text{phacA} \) mutation (Fig. 4, A and B). Finally, wild type mycelia pregrown in glucose and transferred to media containing PhAc secreted 2-hydroxy-PhAc to the culture supernatant (Fig. 5). In agreement with the above in vitro assays, secretion of 2-hydroxy-PhAc was also markedly reduced, but not abolished by the \( \Delta \text{phacA} \) mutation (Fig. 5, A and B). Mycelia were pregrown in glucose minimal medium and transferred to PhAc. Samples were taken at the indicated time-points after the transfer.

**Fig. 4.** \( \text{phacA} \) disruption results in a marked reduction of microsomal PhAc 2-hydroxylase activity. A, a cytochrome P450 monoxygenase catalyzes the incorporation of one of the atoms from dioxygen (as an hydroxyl group) into the aromatic ring of PhAc. The source of electrons for such reaction is a NADPH-cytochrome P450 oxidoreductase, an enzyme that transfers electrons from NADPH through two flavin redox centers (20). In vitro, the activity of the reductase can be monitored by using artificial electron acceptors such as ferricyanide and cytochrome c, as indicated. Shown below are PhAc 2-hydroxylase and NADPH-cytochrome P450 oxidoreductase activities in the microsomal fractions of \( \Delta \text{phacA} \) and \( \text{phacA}^+ \) mycelia. Formation of 2-hydroxy-PhAc was monitored with a chemical method (17). B, HPLC analysis of PhAc 2-hydroxylase in the above microsomal fractions. The positions of standards were indicated by roman numbers as follows: I, 2,5-dihydroxy-PhAc; II, 3,4-dihydroxy-PhAc; III, 4-hydroxy-PhAc; IV, 3-hydroxy-PhAc; V, 2-hydroxy-PhAc; VI, PhAc. The retention time for authentic 2-hydroxy-PhAc was 11.82 min, whereas the product formed in the complete \( \text{phacA}^+ \) and \( \Delta \text{phacA} \) reactions showed retention times of 11.81 and 11.86 min, respectively.

**Fig. 5.** Reduced secretion of 2-hydroxyphenylacetate in a \( \Delta \text{phacA} \) strain. Culture supernatants of \( \text{phacA}^+ \) (circles) and \( \Delta \text{phacA} \) (triangles) strains were assayed for the presence of 2-hydroxy-PhAc. Mycelia were pregrown in glucose minimal medium and transferred to PhAc. Samples were taken at the indicated time-points after the transfer.

PhAc and NADPH, we detected the formation of a monohydroxylated PhAc derivative using a chemical detection method ((Fig. 4A; see “Experimental Procedures”). Direct HPLC analysis of the reaction mixture showed that this compound was 2-hydroxy-PhAc and that its formation was absolutely dependent on the presence of both PhAc and NADPH (Fig. 4B). This showed that A. nidulans microsomes contain a phenylacetate ortho-hydroxylating activity. This activity was markedly and reproducibly reduced but not abolished by the \( \Delta \text{phacA} \) mutation (Fig. 4, A and B). Finally, wild type mycelia pregrown in glucose and transferred to media containing PhAc secreted 2-hydroxy-PhAc to the culture supernatant (Fig. 5). In agreement with the above in vitro assays, secretion of 2-hydroxy-PhAc was also markedly reduced, but not abolished by the \( \Delta \text{phacA} \) mutation (Fig. 5). All these data, together with the growth characteristics of the \( \Delta \text{phacA} \) strain (see above), strongly support the conclusion that \( \text{phacA} \) encodes a phenylacetate 2-hydroxylase and that a second, minor enzyme showing this activity is present in A. nidulans microsomes (see “Discussion”).

**PhacA Disruption Results in Increased Penicillin Production**—Penicillin-producing filamentous fungi use PhAc (activated as a CoA thioester) as a precursor for penicillin G biosynthesis. PhAc is exchanged with the L-aminoadipyl moiety of isopenicillin N to yield penicillin G in a reaction catalyzed by acyl-CoA:isopenicillin N-acyltransferase (see Ref. 21 for review). PhAc cannot be synthesized by fungi, and therefore it has to be fed to penicillin cultures. We reasoned that the PhAc degradation pathway would compete with the penicillin biosynthetic pathway for PhAc, suggesting that interrupting the initial step of the degradation pathway may improve the incorporation of PhAc into penicillins. This prediction was confirmed with the two above \( \Delta \text{phacA} \) strains, which in independent experiments reproducibly showed a marked elevation in penicillin production over the wild type. Fig. 6 shows one such
**DISCUSSION**

We have analyzed the “upper pathway” of PhAc catabolism (i.e. the conversion of PhAc to homogentisate, Fig. 1) in the filamentous fungus *A. nidulans*. Mutational and molecular evidence strongly indicates that this conversion proceeds via two sequential hydroxylating steps, the first of which is a 2-hydroxylolation of the aromatic ring catalyzed by a cytochrome P450 monoxygenase encoded by the *phacA* gene. First, we have isolated a class of mutations preventing growth on PhAc but allowing growth on 2-hydroxy-PhAc or homogentisate as sole carbon source. Second, we describe a gene, denoted *phacA*, whose inactivation results in the same phenotype as the above mutations. This *phacA* mutation does not complement with a prototypical classical mutation of the above class, indicating that both affect the same gene. Third, *phacA* encodes a CYP (cytochrome P450 monoxygenase). CYP enzymes are usually involved in a variety of biosynthetic and catabolic hydroxylating reactions (5, 6), in agreement with the predicted PhAc function. Fourth, *phacA* microsomal extracts catalyze the PhAc- and NADPH-dependent synthesis of 2-hydroxy-PhAc. By contrast, a mutant *ΔphacA* microsomal fraction is markedly deficient in this reaction. An *A. niger* microsomal, NADPH-dependent phenylacetate-2-monoxygenase activity previously reported by others (17) is possibly encoded by a *phacA* homologue. In agreement with our Northern analysis, such activity was detected in phenylacetate-grown cells and absent from glucose-grown cells (17).

The *ΔphacA* mutation constructed here is almost certainly a null mutation, which strongly suggested that the residual PhAc 2-hydroxylase activity that we detected with *ΔphacA* microsomal extracts is encoded by a different gene. We have identified the gene (denoted *pshA*) encoding this minor activity. In agreement with our prediction, a double *ΔphacA ΔpshA* mutation abolished microsomal PhAc 2-hydroxylation. A *ΔpshA* strain grows on PhAc but does not grow on 3-hydroxy-PhAc (which in the wild type is also catabolized through homogentisate), indicating that *pshA* encodes a 3-hydroxy-PhAc 6-hydroxylase (i.e. and ortho-hydroxylase), converting 3-hydroxy-PhAc to 2,5-dihydroxy-PhAc. This enzyme has been previously described in the fungus *Trichosporon cutaneum*, which converts PhAc to homogentisate through sequential hydroxylation of positions 3 and 6 of the ring (22).

Higher eukaryotes have multiple cytochrome P450 monoxygenases catalyzing a variety of oxidative reactions. Such abundance is not found in the microbial world. For example, only three CYP genes are found in the genome of *S. cerevisiae* (23). The marked metabolic versatility of filamentous fungi would suggest a greater variety of CYP enzymes in their proteomes. In addition to *phacA*, four *stc* genes of the *A. nidulans* sterigmatocystin biosynthetic cluster encode CYP enzymes (24). The closely related organism *A. niger* has a very specific benzoate-4 hydroxylase enzyme, encoded by the *bpHA* gene (25). Notably, PhAc and BpHA each define a new CYP family, suggesting that metabolically versatile saprophytic fungi may represent an as yet unexplored source of variability for CYP enzymes catalyzing novel metabolic reactions.

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