Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9132–9136, September 1995
Genetics

Fungal metabolic model for human type I hereditary tyrosinaemia

(*Aspergillus*/alkaptonuria/fumarylacetoacetate hydrolase/phenylalanine)

JOSÉ M. FERNÁNDEZ-CAÑÓN AND MIGUEL A. PEÑALVA

Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, 28006, Madrid, Spain

Communicated by Norman H. Giles, The University of Georgia, Athens, GA, May 22, 1995

ABSTRACT **Type I hereditary tyrosinaemia (HT1) is a severe human inborn disease resulting from loss of fumarylacetoacetate hydrolase (Fah). Homozygous disruption of the gene encoding Fah in mice causes neonatal lethality, seriously limiting use of this animal as a model. We report here that *fahA*, the gene encoding Fah in the fungus *Aspergillus nidulans*, encodes a polypeptide showing 47.1% identity to its human homologue. *fahA* disruption results in secretion of succinylacetone (a diagnostic compound for human type I tyrosinaemia) and phenylalanine toxicity. We have isolated spontaneous suppressor mutations preventing this toxicity, presumably representing loss-of-function mutations in genes acting upstream of *fahA* in the phenylalanine catabolic pathway. Analysis of a class of these mutations demonstrates that loss of homogentisate dioxygenase (leading to alkaptonuria in humans) prevents the effects of a Fah deficiency. Our results strongly suggest human homogentisate dioxygenase as a target for HT1 therapy and illustrate the usefulness of this fungus as an alternative to animal models for certain aspects of human metabolic diseases.**

Genetic blocks in phenylalanine catabolism cause several human inborn diseases (see Fig. 1), the most severe of which is type I hereditary tyrosinaemia (HT1), resulting from loss of fumarylacetoacetate hydrolase (Fah; EC 3.7.1.2) (1, 2). Human Fah deficiency causes accumulation of toxic phenylalanine/tyrosine catabolites in tissues of affected patients and secretion into urine of succinylacetone, a diagnostic compound for the disease resulting from spontaneous conversion of fumarylacetoacetate and maleylacetoacetate (2, 3). Possibly as a result of toxicity caused by the above phenylalanine/tyrosine catabolites, HT1 patients die from hepatic failure or primary liver cancer.

Animal models are often used to evaluate therapeutic strategies. However, the complete loss of Fah in mice causes neonatal lethality (4–7), limiting the usefulness of mice disrupted for the *Fah* gene as a model. Moreover, very low levels of Fah are sufficient to restore a nearly wild-type phenotype in these mutant mice (6), also limiting the utility of a partial deficiency.

The ascomycete *Aspergillus nidulans* shows considerable metabolic versatility. For example, it grows on phenylalanine or phenylacetate (PhOAc) as sole carbon sources. Mutational analysis of PhOAc catabolism (unpublished data) indicates that it proceeds through two sequential aromatic ring hydroxylations to yield 2,5-dihydroxyphenylacetate (homogentisate, Fig. 1). Homogentisate is usually catabolized by cleavage of the aromatic ring to yield ultimately fumarylacetoacetate, which is split by Fah into fumarate and acetoacetate. Although phenylalanine catabolism has not been investigated in any detail in *A. nidulans*, in humans phenylalanine (and tyrosine) are also catabolized through homogentisate to fumarylacetoacetate (Fig. 1). If the *A. nidulans* pathway were similar, Fah would be required for the catabolism of both phenylalanine and PhOAc by the fungus (Fig. 1). We report here that this is the case. Characterization of *fahA*, the *A. nidulans* gene encoding Fah, revealed a notable amino acid sequence conservation between the fungal and human enzymes.* As in humans, disruption of *fahA* results in phenylalanine toxicity and secretion of succinylacetone. These similarities prompted us to use classical suppressor analysis of the phenylalanine toxicity phenotype associated with *fahA* disruption to investigate possible enzyme targets for HT1 therapy. We found that loss of homogentisate dioxygenase prevents phenylalanine toxicity. Loss of this enzyme in humans causes alkaptonuria (8), a much less severe disease than HT1.

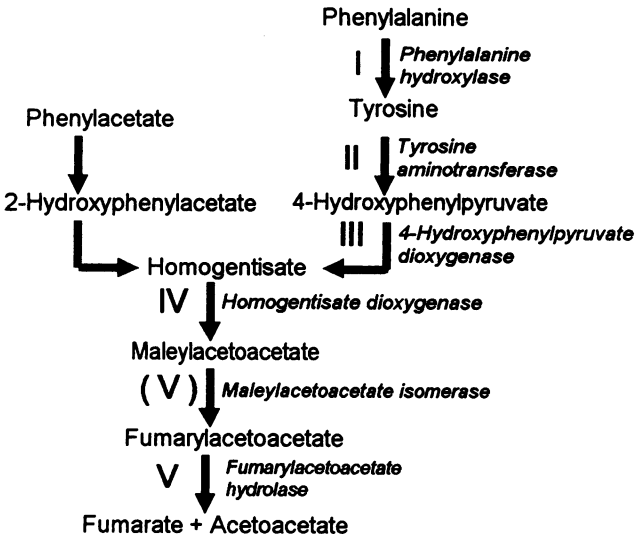

FIG. 1. PhOAc (*A. nidulans*) and phenylalanine (human and *A. nidulans*) pathways. The absence of the indicated enzymes results in the following human diseases: I, classical phenylketonuria and hyperphenylalaninaemias; II, type II tyrosinaemia (Richner–Hanhart syndrome); III, type III tyrosinaemia; IV, alkaptonuria; and V, hereditary tyrosinaemia type I.

MATERIALS AND METHODS

Strains and Media. *A. nidulans biA1* was used as the wild-type strain and source of RNA. All strains were cultured on appropriately supplemented minimal medium (9) with ammonium tartrate as the nitrogen source and carbon sources added as indicated.

***fahA* Disruption.** The *fahA* gene of an *A. nidulans biA1 methG1 argB2* strain was disrupted by transformation to arginine prototrophy by standard procedures (10) with a genomic *Eco*RI fragment containing a mutant *fahA* gene in which a 3.2-kb fragment containing the *argB*$^+$ gene had replaced the sequence between codons 188 and 286 of the *fahA* open reading frame. Several transformants were unable to grow on

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Fah, fumarylacetoacetate hydrolase; HT1, type I hereditary tyrosinaemia; PhOAc, phenylacetate.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L41670).

phenylalanine (or PhOAc) as the sole carbon source. Southern hybridization experiments with *argB*- and *fahA*-specific probes confirmed that in two selected clones the wild-type *fahA*$^+$ allele had been replaced by the *fahA*::*argB*$^+$ fusion.

Subtractive cDNA Hybridization, Library Screening, and Transcript Analysis. *Aspergillus fahA* cDNA clones were isolated by differential screening of a phage λgt10 library constructed with mRNA from PhOAc-induced cells. A cDNA probe corresponding to transcripts induced by PhOAc was obtained by subtractive cDNA hybridization ("cDNA subtraction") essentially as described (11, 12). Briefly, mycelia of the wild-type strain were grown at 37°C in glucose-minimal medium; 2 hr after glucose was consumed (as determined enzymatically with a Boehringer Mannheim kit), 10 mM PhOAc was added to the culture, which was shaken further for 1 hr. After this time, mycelia were frozen in liquid nitrogen and used to prepare poly(A)$^+$ RNA, which was reverse-transcribed with avian myeloblastosis reverse transcriptase (Promega). This cDNA population was sequentially hybridized with an excess of poly(A)$^+$ RNA from mycelia harvested at the time at which glucose was exhausted and with an excess of poly(A)$^+$ RNA from noninduced mycelia (i.e., mycelia cultured in a similar way but omitting induction with PhOAc). Hybrids were removed after each hybridization by chromatography on hydroxyapatite at 68°C. The subtracted cDNA probe was used in library screening. cDNA clones corresponding to PhOAc-induced transcripts were identified by its hybridization with this probe ("plus" probe) and lack of hybridization with a cDNA probe ("minus" probe) corresponding to poly(A)$^+$ RNA from noninduced mycelia. Genomic clones were obtained from a λEMBL4 library from the wild-type strain. For RNA analysis, mycelia grown in minimal medium containing 0.3% glucose for 16 hr at 37°C were transferred to minimal medium with no carbon source added. After a 1-hr incubation, the culture was divided equally into 13 parts and different carbon sources were added as follows: glucose at 1% (wt/vol) or all aromatic compounds and glutamate at 10 mM or KOAc at 30 mM. These secondary cultures were incubated for 1 hr at 37°C. Mycelia were then harvested and used to isolate RNA (13), which was analyzed by Northern blot hybridization.

Enzyme Assays. Mycelia were grown as for RNA isolation, transferred to minimal medium containing 10 mM PhOAc, and incubated for a further 3 hr. Washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7) and lysed by sonication. Cleared lysates (50 μl) were used in enzyme assays, which were made in the above buffer containing 1 mM ascorbate, 50 μM $FeSO_4$, and 0.25 mM homogentisate. Homogentisate dioxygenase activity was determined spectrophotometrically after formation of maleylacetoacetate at 330 nm (14). When this reaction stopped (with >80% of the substrate converted), 100 μM reduced glutathione was included in the reaction mixtures to allow conversion of maleylacetoacetate to fumarylacetoacetate (15). Fah was then assayed by monitoring the decrease in A_{330} resulting from fumarylacetoacetate hydrolysis to fumarate and acetoacetate (16). Acetoacetate formation was verified enzymatically by using NADH-dependant 3-hydroxybutyrate dehydrogenase (Boehringer Mannheim), which was assayed by following the decrease in A_{340} in the presence of 200 μM NADH (17).

GC/MS and HPLC Analyses of Culture Filtrates. For GC/MS analysis, mycelia of *fahA*$^+$ and *fahA*::*argB*$^+$ strains were pregrown as for RNA isolation, transferred to minimal medium containing 25 mM phenylalanine, and incubated for a further 20 hr. Culture filtrates were acidified and extracted with ether. Extracted compounds were treated with bis(trimethylsilyl)trifluoroacetamide for 20 min at 80°C in the presence of 4 μg of ethylvanillin as internal standard. Trimethylsylyl derivatives were analyzed by GC/MS with a SPB1 column (30 m length, 0.25 mm diameter) and an ion-trap detector. The temperature was increased from 100°C to 250°C at a rate of 4°C per min.

For HPLC analysis, cells pregrown as above were transferred to minimal medium containing 10 mM PhOAc or 25 mM phenylalanine and further incubated for 8 hr. Culture supernatants were clarified through 0.45 μM filters and injected into a Nucleosil 300-5 C_{18} column (250 × 4 mm) coupled to a 11 × 4 mm precursor column of this support. An 80:20 (vol/vol) ratio of 50 mM sodium phosphate, pH 6.5/methanol was used as mobile phase at 0.5 ml per min.

RESULTS

Cloning and Characterization of *fahA*, the *A. nidulans* Gene Encoding Fah. Fungal catabolic pathways are usually under the control of specific transcriptional activators, ensuring that

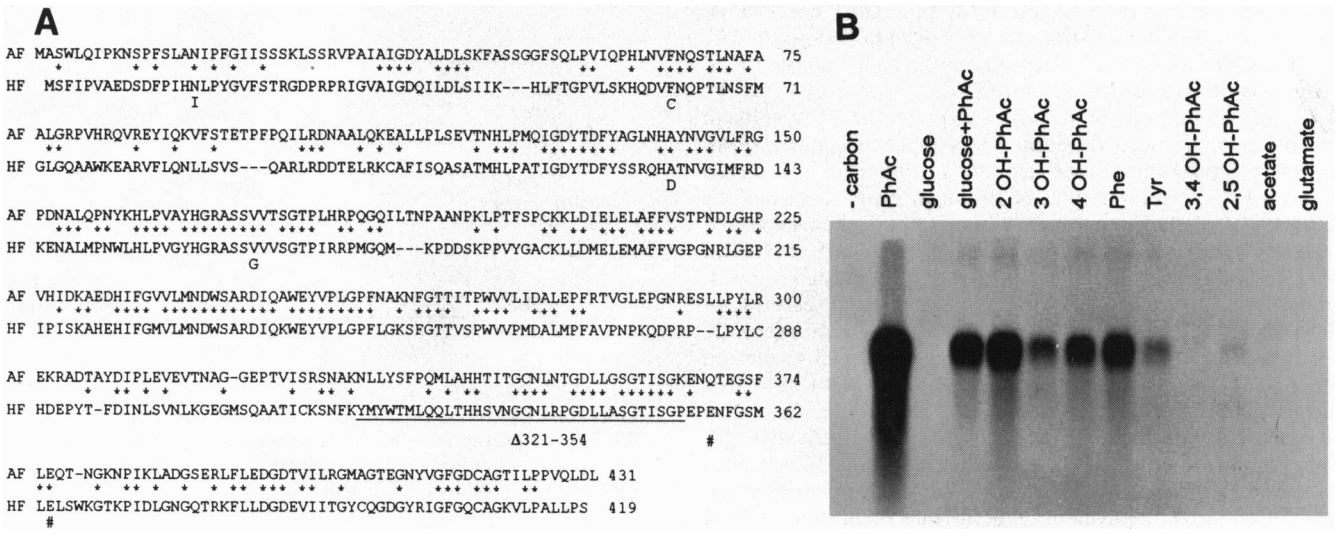

FIG. 2. (*A*) Amino acid sequence of *Aspergillus* FahA (AF) and similarity to that of its human homologue (HF) (18, 19) is shown. The alignment was obtained with BESTFIT (Genetics Computer Group package). Asterisks denote identities. Mutational changes, all of which have been reported in HT1 patients, are indicated below the human sequence as follows: I, Asn-16 → Ile (20); C, Phe-62 → Cys (21); D, Ala-134 → Asp (19); G, Val-166 → Gly (22); #, Glu-357 → stop codon and Glu-364 → stop codon (22); and underlining, deletion of residues 321–354 (22). (*B*) Transcription of *fahA* is induced by PhOAc or phenylalanine. Northern blot analysis shows *fahA* transcript levels after transfer of mycelia to the indicated carbon sources. The probe was a ^{32}P-labeled 1.35-kb cDNA containing the complete *fahA* open reading frame. Equal loading in each lane was determined by hybridization with an actin probe (not shown).

expression of structural genes occurs only when an appropriate inducer is available. Accordingly, we have used a subtractive cDNA hybridization procedure to isolate clones corresponding to *A. nidulans* transcripts induced by PhOAc. Sequencing of several overlapping cDNA clones of a highly represented transcript revealed an open reading frame putatively encoding a 431-residue polypeptide. A data-base search detected high amino acid sequence similarity between the predicted product and mammalian Fah polypeptides. For example, the derived amino acid sequence shows 47.1% identity to human Fah (18, 19) (Fig. 2*A*), strongly indicating that this open reading frame encodes its fungal counterpart (see below). Therefore, the gene was designated *fahA*. Amino acid identity is particularly high in certain regions of the protein (Fig. 2*A*) that contain residues crucial for normal human Fah activity (20–22). The nucleotide sequence of genomic clones corresponding to this open reading frame was found to be colinear with that deduced from cDNA clones, indicating that, in contrast to its human homologue (19), the fungal gene does not contain introns.

Northern analysis showed that fahA transcript levels are highly inducible by PhOAc and that transcription is negligible in its absence (Fig. 2*B*). Also phenylalanine substantially induced transcript levels, strongly suggesting that catabolism of both PhOAc and phenylalanine proceeds, as does mammalian phenylalanine catabolism, through homogentisate and fumarylacetoacetate (see Fig. 1) and that the true inducer of *fahA* transcription is a catabolite common to the phenylalanine and PhOAc pathways. Glutamate and acetate did not increase transcript levels (Fig. 2*B*), ruling out induction by a gluconeogenic intermediate(s). Remarkably, the gene was not fully repressed by glucose.

Disruption of *fahA* Results in Secretion of Succinylacetone and Prevents Growth on Phenylalanine or PhOAc. A disrupted *fahA* allele was generated by reverse genetics (Fig. 3*A*). The disrupted strain (Δ*fahA*) grows normally on medium containing 0.05% lactose (Fig. 3*B*) or 1% glucose (not shown) but is unable to grow on medium containing either PhOAc or phenylalanine as the sole carbon source. No Fah activity was detected in mycelial extracts from a Δ*fahA* strain pregrown in glucose medium and transferred to medium containing PhOAc (Fig. 3*C*). These growth conditions resulted in high levels of Fah in a *fahA*$^+$ strain. In contrast, homogentisate dioxygenase activity was induced in the disruptant to (at least) wild-type levels upon transfer. These results, together with amino acid sequence data, establish that *fahA* encodes *A. nidulans* Fah and demonstrate that phenylalanine and PhOAc catabolism in *Aspergillus* takes places through Fah-mediated hydrolysis of fumarylacetoacetate.

HT1 (a deficiency in human Fah) specifically results in accumulation of succinylacetone (presumably originated spontaneously from fumarylacetoacetate) in the urine of affected patients (2). This compound is absent in urine from healthy subjects or from patients with diseases of phenylalanine/tyrosine metabolism other than HT1 (2, 3). GC/MS analysis of filtrates of cultures transferred to broth containing phenylalanine showed that the *fahA* disruption resulted in secretion of several phenylalanine metabolites that were absent from the *fahA*$^+$ filtrate (Fig. 4), including three peaks whose elution times and mass spectra were identical to those of authentic succinylacetone (Fig. 4 *Lower Right*). These peaks probably represent steric isomers of the succinylacetone derivative (2). This provides further evidence that the disrupted gene encodes *A. nidulans* Fah and illustrates the remarkable similarity of the consequences of equivalent genetic blocks in human and fungal phenylalanine catabolism.

***fahA* Disruption Results in Phenylalanine (and PhOAc) Toxicity.** We noted the complete absence of growth of Δ*fahA* in minimal medium containing phenylalanine (or PhOAc) (Fig. 3*B*). This is in contrast to the residual growth of *A. nidulans* in the absence of a carbon source, indicating that, analogous to the situation in humans, the absence of Fah leads

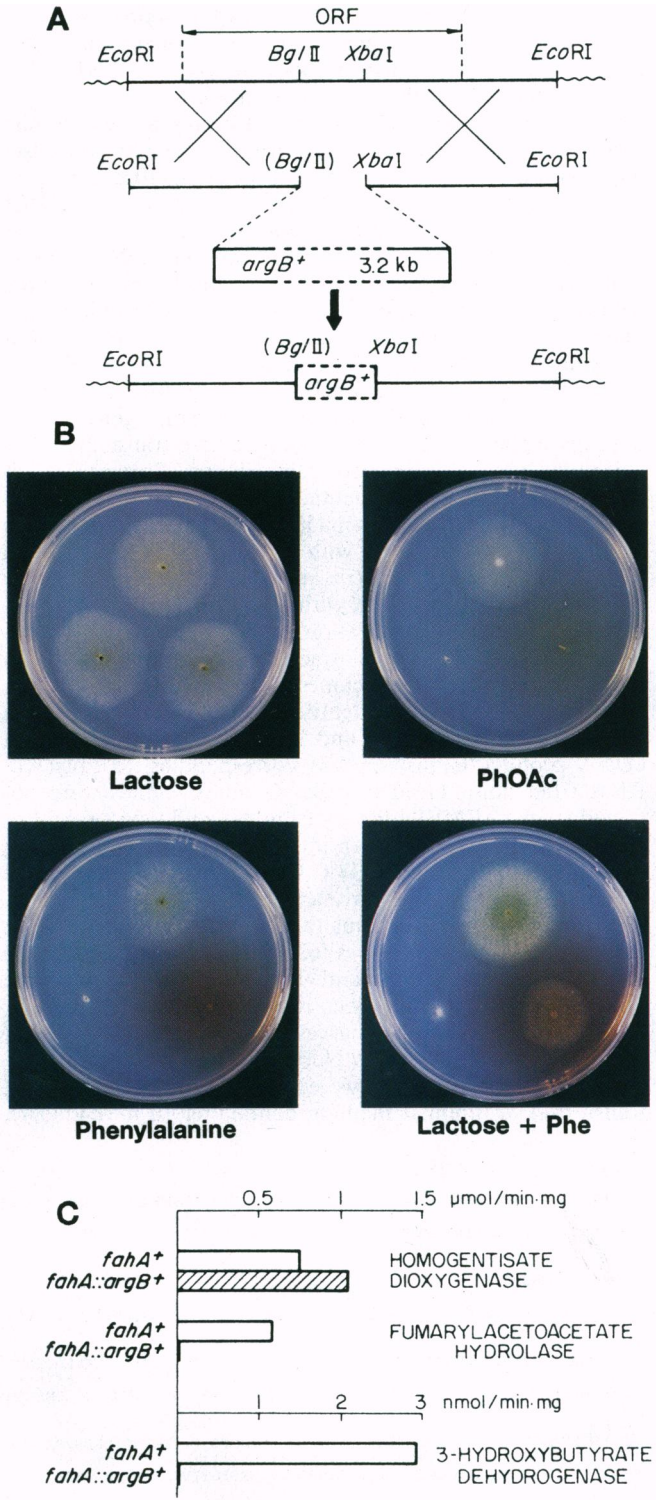

FIG. 3. Disruption of the *Aspergillus fahA* gene and suppressor mutations. (*A*) A linear fragment containing a disrupted copy of the *fahA* gene (*fahA*::*argB*$^+$) was used to replace the endogenous *fahA*$^+$ allele. Two disruptant clones were selected and shown to have identical phenotypes. (*B*) Growth phenotypes of the disrupted strain and of a derivative carrying a suppressor mutation. Conidiospores of the *fahA*$^+$ (*B Upper*), *fahA*::*argB*$^+$ (*B Lower Left*) and *fahA*::*argB*$^+$ *suAfah* (=*hmgA*$^-$, *vide infra*) (*B Lower Right*) strains were inoculated on minimal medium plates with the indicated carbon sources (lactose at 0.05%, phenylalanine at 25 mM, or PhOAc at 10 mM). Plates were photographed after a 3-day incubation at 37°C. (*C*) Homogentisate dioxygenase and Fah assays in extracts from *fahA*$^+$ and *fahA*::*argB*$^+$ strains. Acetoacetate formation was verified enzymatically.

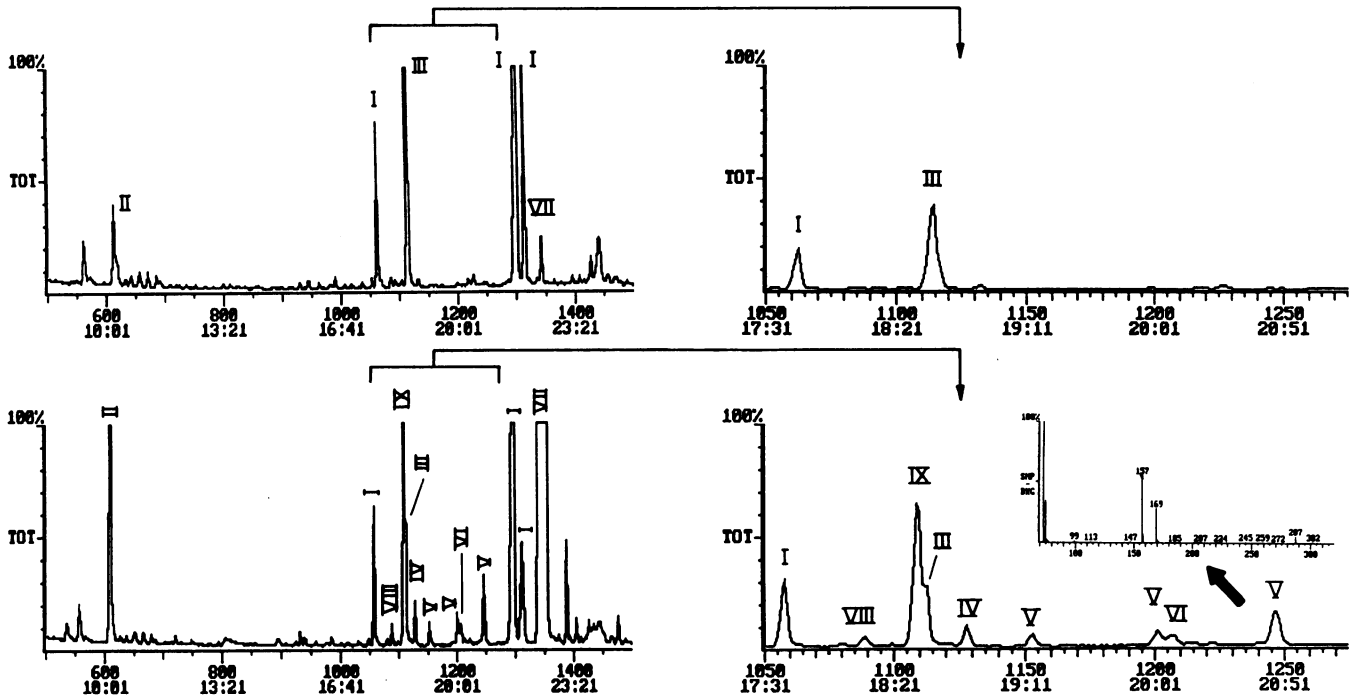

FIG. 4. Detection of succinylacetone in culture filtrates of the *fahA*::*argB*$^+$ strain. Ether-extractable compounds in culture supernatants of *fahA*::*argB*$^+$ (*Lower*) and wild-type (*Upper*) strains were analyzed (as trimethylsilyl derivatives) by GC-MS. (*Right*) Expanded regions of the chromatograms. Detector response is plotted vs. retention times. The three peaks corresponding to succinylacetone (indicated with V), detected only in the culture filtrate of the disrupted strain, were identified by their retention times and mass spectra, which were indistinguishable of those of authentic succinylacetone (3). Only the mass spectrum of the major peak is shown in *Bottom Right Inset*. All other identified peaks were assigned to a compound by computer comparison of their mass spectra with those in the NBS library and are denoted by roman numbers as follows: II, PhOAc; III, ethylvanillin (internal standard); IV, phenyllactate; VI, 4-OH-PhOAc; VII, phenylpyruvate; VIII, 2-OH-PhOAc; IX, an unidentified metabolite of PhOAc; and I, three unidentified peaks present in all chromatograms.

to accumulation of toxic metabolites (perhaps succinylacetone, see above). Plate assays (Fig. 3*B*) in the presence of a derepressing concentration of lactose and phenylalanine (or PhOAc; not shown) confirmed the toxicity: the wild-type strain grows and conidiates well in the presence of this combination of carbon sources, but growth of the disruptant is severely impaired (Fig. 3*B*).

Suppressor Mutations Preventing Phenylalanine Toxicity Associated with *fahA* Disruption Result in Loss of Homogentisate Dioxygenase. Vigorously growing sectors appeared frequently from Δ*fahA* colonies on minimal medium containing lactose and phenylalanine or PhOAc after long incubation times. These sectors result from a second, spontaneous mutation suppressing phenylalanine or PhOAc toxicity or both. Since inhibitors of 4-hydroxylphenylpyruvate dioxygenase can alleviate the effects of human Fah deficiency (23), we reasoned that earlier blocks in the pathway might prevent phenylalanine and PhOAc toxicity in a Δ*fahA* background. Therefore, we isolated a number of these suppressors and found two classes of mutations. One class prevents toxicity only on the selection medium (i.e., on medium with lactose and phenylalanine or lactose and PhOAc) and presumably identifies genes involved in degradation of the corresponding aromatic compound to homogentisate. Mutations in the second class are phenotypically homogeneous. These mutations, designated *suAfah*, prevent the effects of Fah deficiency on both lactose/phenylalanine- and lactose/PhOAc-containing media, indicating that they are defective in one of the two common steps of the pathway before Fah (Fig. 1). Because defects in maleylacetoacetate isomerase would cause similar effects to those in Fah (2), this second class is likely to result in loss of homogentisate dioxygenase. Notably, *suAfah* mutations result in secretion of a red pigment (Fig. 3*B*), which is turned dark-brown after long incubation times (not shown). Alkaptonuria (a deficiency of human homogentisate dioxygenase) results in secretion into the urine of homogentisate (which is converted to a dark brown pigment upon oxidation by air) and ochronosis (i.e., pigmentation by polymers of homogentisate) of connective tissues. Therefore, these data strongly suggested that these *suAfah* mutations represented the loss-of-function class in the homogentisate dioxygenase gene. This gene, designated *hmgA*, has also been cloned by the subtractive cDNA hybridization procedure. Its identification has been established by targeted disruption and by expression in *Escherichia coli* of this protein product, which shows homogentisate dioxygenase activity (26).

Self-induced correction of HT1 deficiency by reversion of Fah point mutations has been described (24). Our *suAfah* suppressor mutations do not result from reversion. First, our suppressed *fahA* mutation is a gene disruption–deletion (Fig. 3*A*). Second, genetic analysis (see below) showed that *suAfah* mutations are *hmgA* alleles (not *fahA* alleles). In contrast, the following arguments strongly indicate that *suAfah* mutations represent the loss-of-function class in *hmgA*: (*i*) while the Δ*fahA* strain shows wild-type levels of homogentisate dioxygenase, a Δ*fahA suAfah* double mutant strain completely lacks this activity (Fig. 5*A*) but not maleylacetoacetate isomerase (data not shown); (*ii*) HPLC analyses of culture filtrates (Fig. 5*B*) demonstrated that this double mutant strain secretes homogentisate into the medium, as would be expected from loss of homogentisate dioxygenase; (*iii*) targeted disruption of *hmgA* results in a phenotype indistinguishable from that of *suAfah* mutations; (*iv*) *suAfah* mutations and a *hmgA*::*argB*$^+$ gene disruption are recessive in diploids; and (*v*) nine independent *suAfah* mutations having an identical phenotype do not complement in diploids with *hmgA*::*argB*$^+$ and are therefore allelic to this disruption.

DISCUSSION

The *A. nidulans* phenylalanine pathway is notably similar to its mammalian counterpart. This similarity is reflected at the protein

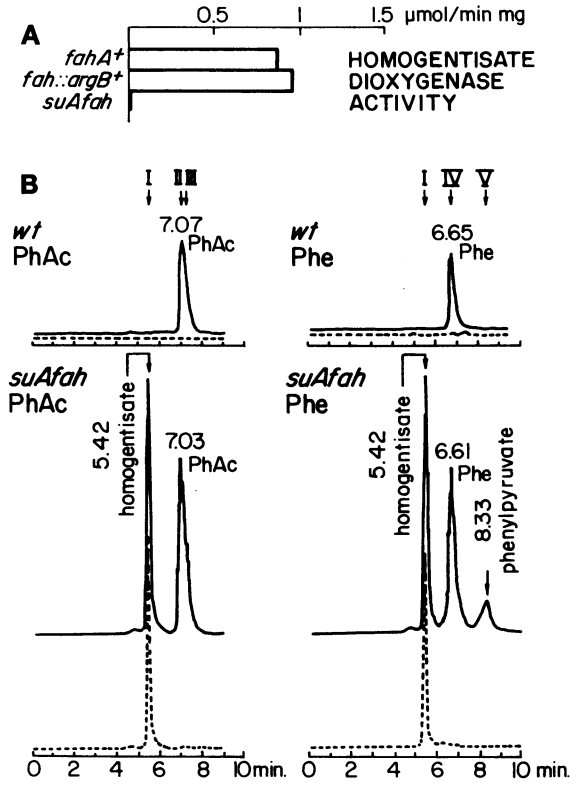

FIG. 5. (*A*) Homogentisate dioxygenase assays in extracts of a wild-type strain, of a *fahA*::*argB*$^+$ strain, and of a double mutant strain, *suAfah fahA*::*argB*$^+$. (*B*) HPLC analysis of culture filtrates of the wild-type (*B Upper*) and a *suAfah fahA*::*argB*$^+$ strain (*B Lower*). (*B Left*) Cultures transferred to PhOAc-containing minimal medium. (*B Right*) Cultures transferred to phenylalanine-containing minimal medium. Absorbance was monitored at 220 nm (continuous line) or 290 nm (discontinuous line), a wavelength at which homogentisate presents a characteristic absorbance maximum. Retention times of standards are indicated by arrows as follows: I, homogentisate; II, 2-OH-PhOAc; III, PhOAc; IV, phenylalanine; and V, phenylpyruvate.

sequence level. For example, the amino acid sequence of *A. nidulans* Fah is 47% identical to its human homologue, possibly reflecting toxicity caused by loss of this activity. Sequence comparison between the mammalian and fungal enzymes will be helpful in establishing structure–function relationships.

Deficiency of human Fah causes HT1, a severe metabolic disease (1, 2). Affected children frequently die within a year after birth (1). The only alternative to liver transplantation is the administration of a herbicide compound that inhibits the upstream enzyme 4-hydroxyphenylpyruvate dioxygenase (23). The above similarity prompted us to investigate the consequences of equivalent genetic blocks in fungal and human phenylalanine catabolism. Disruption of *fahA* results in phenylalanine toxicity (a conditional phenotype, as the disruptant strain grows normally in the absence of phenylalanine) and secretion of succinylacetone (as shown by GC/MS analysis of culture filtrates). The presence of succinylacetone in human urine is diagnostic for the disease.

The mouse gene encoding Fah has been characterized (4). Homozygous disruption or deletion of the gene causes neonatal lethality (4–7). This is a serious limitation for mice as an animal model for HT1. In the light of the above similarities, we reasoned that the *A. nidulans* strain disrupted for *fahA* could be helpful as a model of at least some aspects of the disease, such as the toxicity caused by certain phenylalanine catabolites. Therefore, we used classical suppressor analysis of phenylalanine toxicity and established that loss of homogentisate dioxygenase prevents the effects of Fah deficiency in *Aspergillus*. Mammalian homogentisate dioxygenase genes are as yet uncharacterized (25). The absence of this enzyme in humans causes alkaptonuria (8), a much less severe defect than HT1. Therefore, we predict that homozygous disruption of the mouse homogentisate dioxygenase gene will prevent neonatal lethality associated with homozygous deletions of the mouse Fah gene (4–7) and propose that in humans alkaptonuria would prevent the severe effects of HT1. Availability of the fungal *hmgA* gene combined with the remarkable similarity between the fungal and mammalian genes reported here for at least one enzyme of phenylalanine catabolism will help to identify their mammalian homologues. Finally, enzyme assays or growth tests with the *A. nidulans* Δ*fahA* strain could be used to screen for inhibitors of homogentisate dioxygenase to be used in therapy.

We thank A. Prieto for GC/MS, J. L. Garcia and M. A. Prieto for advice with HPLC analysis, E. Reoyo for technical assistance, and H. N. Arst, Jr., and G. Morata for comments on the manuscript. This work was supported by the Comisión Interministerial de Ciencia y Tecnología (Spain). J.M.F.-C was funded by a contract with Antibióticos S.A.

1. Goldsmith, L. A. & Laberge, C. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. & Valle, D. (McGraw-Hill, New York), pp. 547–562.
2. Lindblad, B., Lindstedt, S. & Steen, G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4641–4645.
3. Grenier, A., Lescault, A., Laberge, C., Gagné, R. & Mamer, O. (1982) *Clin. Chim. Acta* **123**, 93–99.
4. Klebig, M. L., Russell, L. B. & Rinchik, E. M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1363–1367.
5. Ruppert, S., Kelsey, R., Schedl, A., Schmid, E., Thies, E. & Schütz, G. (1992) *Genes Dev.* **6**, 1430–1443.
6. Kelsey, G., Ruppert, S., Beermann, F., Grund, C., Tanguay, R. M. & Schütz, G. (1993) *Genes Dev.* **7**, 2285–2297.
7. Grompe, M., Al-Dhalimy, M., Finegold, M., Ou, C., Burlingame, T., Kennaway, N. C. & Soriano, P. (1993) *Genes Dev.* **7**, 2298–2307.
8. Garrod, A. E. (1902) *Lancet* **ii**, 1616–1620.
9. Cove, D. J. (1966) *Biochim. Biophys. Acta* **113**, 51–56.
10. Miller, B. L., Miller, K. Y. & Timberlake, W. E. (1985) *Mol. Cell. Biol.* **5**, 1714–1721.
11. Zimmermann, C. R., Orr, W. C., Leclerc, R. F., Barnard, E. & Timberlake, W. E. (1980) *Cell* **21**, 709–715.
12. Sargent, T. D. (1987) *Methods Enzymol.* **152**, 423–432.
13. Espeso, E. A. & Peñalva, M. A. (1992) *Mol. Microbiol.* **6**, 1457–1465.
14. Edwards, S. W. & Knox, W. E. (1955) *Methods Enzymol.* **2**, 292–295.
15. Knox, W. E. & Edwards, S. W. (1955) *Methods Enzymol.* **2**, 295–298.
16. Edwards, S. W. & Knox, W. E. (1955) *Methods Enzymol.* **2**, 299–300.
17. Brock, D. J. H. & Wlliamson, D. H. (1985) in *Methods in Enzymatic Analysis*, ed. Bergmeyer, H. U. (VCH Weinheim, Germany), pp. 69–73.
18. Phaneuf, D., Labelle, Y., Bérubé, D., Arden, K., Cavenee, W., Gagné, R. & Tanguay, R. M. (1991) *Am. J. Hum. Genet.* **48**, 525–535.
19. Labelle, Y., Phaneuf, D., Leclerc, B. & Tanguay, R. M. (1993) *Hum. Mol. Genet.* **2**, 941–946.
20. Phaneuf, D., Lambert, M., Laframboise, R., Mitchell, G., Lettre, F. & Tanguay, R. M. (1992) *J. Clin. Invest.* **90**, 1185–1192.
21. Awata, H., Endo, F., Tanoue, A., Kitano, A., Nakano, Y. & Matsuda, I. (1994) *Biochim. Biophys. Acta* **1226**, 168–172.
22. Grompe, M. & Al-Dhalimy, M. (1993) *Hum. Mutat.* **2**, 85–93.
23. Lindstedt, S., Holme, E., Lock, E. A., Hjalmarson, O. & Strandwick, B. (1992) *Lancet* **340**, 813–817.
24. Kvittingen, E. A., Rootwelt, H., Berger, R. & Brandtzaeg, P. (1994) *J. Clin. Invest.* **94**, 1657–1661.
25. Janocha, J., Wolz, W., Srsen, S., Srsnova, K., Montagutelli, X., Guénet, J. L., Grimm, T., Kress, W. & Müller, C. R. (1994) *Genomics* **19**, 5–8.
26. Fernández-Cañón, J. M. & Peñalva, M. A. (1995) *J. Biol. Chem.*, in press.