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Characterization of a Fungal Maleylacetoacetate Isomerase Gene and Identification of Its Human Homologue*

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We have previously used *Aspergillus nidulans* as a fungal model for human phenylalanine catabolism. This model was crucial for our characterization of the human gene involved in alcaptonuria. We use here an identical approach to characterize at the cDNA level the human gene for maleylacetoacetate isomerase (MAAI, EC 5.2.1.2), the only as yet unidentified structural gene of the phenylalanine catabolic pathway.

We report here the first characterization of a gene encoding a MAAI enzyme from any organism, the *A. nidulans maiA* gene. *maiA* disruption prevents growth on phenylalanine (Phe) and phenylacetate and results in the absence of MAAI activity *in vitro* and Phe toxicity. The MaiA protein shows strong amino acid sequence identity to glutathione *S*-transferases and has MAAI activity when expressed in *Escherichia coli*. *maiA* is clustered with *fahA* and *hmgA*, the genes encoding the two other enzymes of the common part of the Phe/phenylacetate pathways.

Based on the high amino acid sequence conservation existing between other homologous *A. nidulans* and human enzymes of this pathway, we used the MaiA sequence in data base searches to identify human expressed sequence tags encoding its putative homologues. Four such cDNAs were sequenced and shown to be encoded by the same gene. They encode a protein with 45% sequence identity to MaiA, which showed MAAI activity when expressed in *E. coli*.

Human MAAI deficiency would presumably cause tyrosinemia that would be characterized by the absence of succinylacetone, the diagnostic compound resulting from fumarylacetoacetate hydrolase deficiency in humans and fungi. Culture supernatants of an *A. nidulans* strain disrupted for *maiA* are succinylacetone-negative but specifically contain *cis* and/or *trans* isomers of 2,4-dioxohept-2-enoic acid. We suggest that this compound(s) might be diagnostic for human MAAI deficiency.

The catabolism of phenylalanine and tyrosine in humans is both of intrinsic and clinical interest. The enzymatic steps of this pathway were definitively established in the '50s by the work of Knox and colleagues (see Fig. 1*A*; Ref. 1). However, two of its structural genes remained uncharacterized. We recently used a novel approach based on the development of a fungal model to characterize one of them (2–4). Here we report our successful application of this approach to the characterization of the other and address by reverse genetics the consequences of the corresponding enzyme deficiency in our model organism.

An enzyme deficiency in any of the steps of this pathway causes in humans a known metabolic disease. For example, a deficiency in phenylalanine hydroxylase causes phenylketonuria (reviewed by Scriver *et al.* (5)). Enzyme deficiencies in four other steps (those labeled as II, III, and VI in Fig. 1*A*) cause different hypertyrosinemias (reviewed by Mitchell *et al.* (6)), and absence of homogentisate dioxygenase (IV, see Fig. 1*A*) causes alkaptonuria (4, 7). Although the historical interest in the later is notable as it enabled Archibald Garrod to coin the term of "inborn error of metabolism" (8, 9), the gene had not been characterized until recently (3, 4, 10). Crucial for the isolation and characterization of this gene was our establishment of a fungal model for human phenylalanine catabolism based on the filamentous ascomycete *Aspergillus nidulans* (2). We cloned its homogentisate dioxygenase gene (the first gene encoding this enzyme identified for any organism) and used its derived amino acid sequence as a probe to identify in similarity searches of the human expressed sequence, tag data base (EST)[1] cDNAs encoding its human homologue (3).

Type 1 hereditary tyrosinemia (HT1, hepatorenal tyrosinemia, McKusick 276700) is the most severe disease in human Phe catabolism, affecting liver, kidney, and peripheral nerves. HT1 patients surviving infancy develop chronic liver disease with a high incidence of hepatocellular carcinoma (6). HT1 results from fumarylacetoacetate hydrolase (FAAH) deficiency (11). It is generally accepted that fumarylacetoacetate and its spontaneous reaction product, succinylacetone (the diagnostic compound of the disease), are toxic due to their considerable reactivity with key cellular molecules (6, 11), and fumarylacetoacetate has been shown to be mutagenic in Chinese hamster cells (12). In agreement with this, growth of an *A. nidulans* strain disrupted for the FAAH-encoding gene is prevented by phenylalanine even in the presence of an alternative carbon source (2). succinylacetone is accumulated in culture supernatants of this strain, illustrating the equivalent consequences of a FAAH deficiency in humans and *A. nidulans* (2).

The clinical consequences of a MAAI (MAAI, EC 5.2.1.2; step V in Fig. 1*A*) deficiency in humans are largely unknown. It is predicted that this deficiency should also lead to HT1, as maleylacetoacetate has similar reactivity to fumarylacetoacetate (for example, see Ref. 13). By contrast, it is thought that it

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ001836, AJ001837, AJ001838.

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[1] The abbreviations used are: EST, expressed sequence tag; HT1, human type 1 hereditary tyrosinemia; GSH, reduced glutathione; PhAc, phenylacetate; FAAH, fumarylacetoacetate hydrolase; HGO, homogentisate dioxygenase; MAAI, maleylacetoacetate isomerase; TMS, trimethylsilyl; kb, kilobase(s); GC, gas chromatography; MS, mass spectrometry; ORF, open reading frame; UTR, untranslated region.

should not result in the presence of succinylacetone in plasma and urine, as the latter compound is likely to be formed from succinylacetoacetate resulting from *in vivo* reduction of maleyl and fumarylacetoacetate (6, 11). Succinylacetoacetate is efficiently degraded by FAAH (1), and its hydrolysis would prevent succinylacetoacetone formation. Only one such succinylacetone-negative patient showing type 1 tyrosinemia with nondetectable levels of MAAI but normal levels of FAAH in liver has been described (14).

Mammalian MAAI has been little studied since its original characterization (1, 15, 16), possibly due, among other possible reasons, to the instability of the substrate (6). The gene encoding MAAI has not been cloned from any organism, and it is therefore the only structural gene of the Phe/Tyr degradation pathway that remains uncharacterized, precluding the analysis of the molecular basis of succinylacetone-negative type I tyrosinemia. Here we successfully use our fungal model to identify cDNAs encoding human MAAI. The liver enzyme requires glutathione (1, 15, 16) as does the equivalent bacterial enzyme that has been purified to homogeneity (17). Our characterization of fungal and human MAAI cDNAs revealed strong amino acid sequence identity of their derived protein sequences to glutathione *S*-transferases, in agreement with the proposed mechanism of the isomerization (18). We also extend the work of Edwards and Knox (16) and demonstrate MAAI activity by an *in vitro* complementation assay using extracts from a recombinant fungal strain deficient for MAAI. Notably, we detected no succinylacetone in culture supernatants of this strain.

EXPERIMENTAL PROCEDURES

Fungal Strains, Media, and Growth Conditions—*A. nidulans* strains carried markers in standard use (19). A *biA*1 strain was used as a source of cDNA and wild type protein extracts. The *biA*1, *methG*1, Δ*fahA* strain has been described (2). A *biA*1, *methG*1 strain was used as the wild type in growth tests. Standard media for *A. nidulans* (20) were used for strain maintenance, growth tests, and transformation. Culture conditions inducing high levels of expression of the *fahA*/*maiA*/*hmgA* genes, which were routinely used to grow mycelia for protein extraction, have been described (21).

Identification of the A. nidulans maiA Gene—Genomic *maiA* sequences were identified by Southern analysis of DNA from λEMBL4 clones carrying the *fahA* and *hmgA* genes, using a subtracted cDNA probe representing genes induced by phenylacetate ("plus" probe) and a cDNA probe from glucose-grown mycelia ("minus" probe). A 2.5-kb *Eco*RI fragment contiguous to the *fahA* transcription unit (see Fig. 1*B*) showed strong differential hybridization to the plus probe. In addition, cDNA library screening using the F2 fragment (see Fig. 1*A*) resulted in the isolation, in addition to *fahA* cDNA clones, of a second class of cDNA clones representing *maiA* transcripts. The cDNA library enriched in transcripts induced by phenylacetate and the subtracted plus cDNA probe have been described (2, 3). The *A. nidulans* genomic library was a standard λEMBL4 library constructed from the wild type strain. RNA isolation and Northern analysis followed (22). Equal loading of the different samples was confirmed using an actin probe.

Disruption of the maiA Gene—Transformation followed Tilburn *et al.* (23). For disruption of *maiA* we used a 4.2-kb linear DNA fragment in which the sequence between *maiA* codons 140–226 had been replaced by a 3.2-kb *Xba*I fragment carrying *argB*$^+$. A genomic fragment carrying *maiA* sequences from an *Xba*I site at position −133 (relative to the initiation codon) to an *Xho*I site at position +655 (relative to the stop codon) was subcloned in pBS-SK$^+$ (Stratagene). Substitution of an internal 0.26-kb *Sal*I-*Eco*RI fragment by the above 3.2-kb *A. nidulans* genomic fragment (whose *Xba*I ends had been previously converted to *Eco*RI and *Xho*I) removed *maiA* sequences between codons 140 and 226 to yield pBS-ΔMAI. The transforming fragment was isolated from this plasmid after digestion with *Xba*I and *Xho*I.

Enzyme Assays—Maleylacetoacetate, which is not commercially available, was synthesized enzymatically from homogentisate (1, 15) using homogentisate dioxygenase from *A. nidulans* extracts or from *Escherichia coli* cells overexpressing the human enzyme. The procedure used to obtain mycelial protein extracts from Δ*fahA*, Δ*maiA*, and wild type *A. nidulans* strains and the conditions for the homogentisate dioxygenase reaction have been described (21). For *in vitro* complementation assays, the initial homogentisate concentration was 100–125 μM. Maleylacetoacetate formation was monitored spectrophotometrically at 330 nm. When the reaction reached a plateau (with usually more than 80% of the substrate converted to maleylacetoacetate), 150 μM reduced glutathione was added to allow the MAAI-dependent isomerization of maleylacetoacetate to fumarylacetoacetate (1, 16), which is then a substrate for FAAH. Complementation of Δ*maiA* extracts was used to detect MAAI activity in crude lysates of *E. coli* cells overexpressing fungal or human MAAI, as described in the corresponding figure legends.

Absorption Spectra of Intermediates—Maleylacetoacetate was synthesized in separate reactions using 250–500 μM substrate and an excess of recombinant human HGO. When the reactions reached a plateau, they were stopped and deproteinized after the addition of 0.1 vol of 10% metaphosphoric acid, incubation for 10 min in ice, and centrifugation at 13,000 × *g* for 10 min. The supernatants were neutralized with KOH to pH 7.5–8 and immediately used in *in vitro* reactions in the presence of wild type or mutant *A. nidulans* protein extracts. These reaction mixtures were essentially as for homogentisate dioxygenase but contained GSH as a cofactor for MAAI. Reactions were carried out for 15 min at room temperature, deproteinized with metaphosphoric acid, and neutralized with KOH as above. The absorption spectra of these samples were determined and compared with those of duplicate reaction mixtures for which the final neutralization step was omitted.

Overexpression of Proteins in E. coli—High levels of protein expression were achieved using the pD1 vector (a gift of E. Espeso). This is a modified pET19b (Novagen) derivative that was engineered to introduce a single *Bam*HI site allowing in-frame fusion of the desired coding region to an N-terminal His tag. Details of this vector will be described elsewhere. Proteins overexpressed in this system carry the sequence MGHHHHHHHHHHSSGHIDDDDKHMGS at their amino termini. The MaiA coding region was amplified using the following pair of primers (underlined sequences add or modify restriction sites): 5′-CGGGATCCCCCGCACCGGTCAAGATCTC-3′ (upper) and 5′-CGGAATTCAACACCTAAATTCCGTTGGTG-3′ (lower). The fusion protein contains the complete MaiA sequence with four further extra residues (PAPL) between the above N-terminal tag and the MaiA initiation methionine. The corresponding recombinant plasmid was denoted pD1::MaiA. The human MAAI coding region was amplified using EST 265310 (5′) as template and primers 5′-CAGGGATCCAAGCCCATCCTCTATTCC-3′ (upper) and 5′-CAGGAATTCGGAGCTAGGCCCTC-3′ (lower). The recombinant gene fused the above N-terminal tag to residues 5–216 of the protein. The corresponding plasmid was denoted pD1::HSMAAI. A pD1::HSHGO plasmid (a gift from M. C. Estébanez), driving high level expression of human HGO, will be described elsewhere.

Recombinant plasmids were selected in *E. coli* DH1, purified, and transformed into *E. coli* BLB21(DE3)pLysS. Primary transformants were selected on LB plates containing ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) and directly used to inoculate LB liquid cultures that were grown at 37 °C until A_{600} reached 0.8–0.9 units. Expression of T7 RNA polymerase was induced after the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and further incubation for 2.5 h. 0.5 ml samples were taken before and after induction, and bacteria were collected by centrifugation and resuspended in SDS-polyacrylamide gel electrophoresis loading buffer. Samples were boiled for 3 min before loading appropriate aliquots onto a 12% SDS-polyacrylamide gel alongside Bio-Rad wide-range protein markers. Proteins were detected by Coomassie staining.

For preparation of protein extracts, bacteria from a 50-ml culture were collected by centrifugation, washed in 100 mM potassium phosphate buffer, pH 7.0, resuspended in 4 ml of the same buffer, and lysed by sonication. Lysates were clarified by centrifugation at 10,000 rpm and 4 °C for 20 min in an SS34 rotor. Protein concentrations were estimated by the Bradford assay (24).

GC-MS Analysis of Culture Filtrates—Fungal mycelia pregrown on 0.6% glucose (w/v) as the sole carbon source were transferred to appropriately supplemented minimal medium with 20 mM phenylacetate as the sole carbon source (see Ref. 21) and incubated for 20 h at 37 °C. Culture filtrates were ether-extracted and derivatized with bis(trimethylsilyl)trifluoroacetamide as described (2). TMS derivatives were analyzed by GC-MS in a fused silica capillary column SBP-1 (30 m × 0.25 mm; 0.2-mm film thickness) with a temperature program from 80 to 280 °C (4 °C/min), and a Q-MASS (Perkin-Elmer) mass detector. Identification of peaks was carried out by comparison of sample spectra with reference spectra from the NIST/EPA/NIH mass spectral data base.

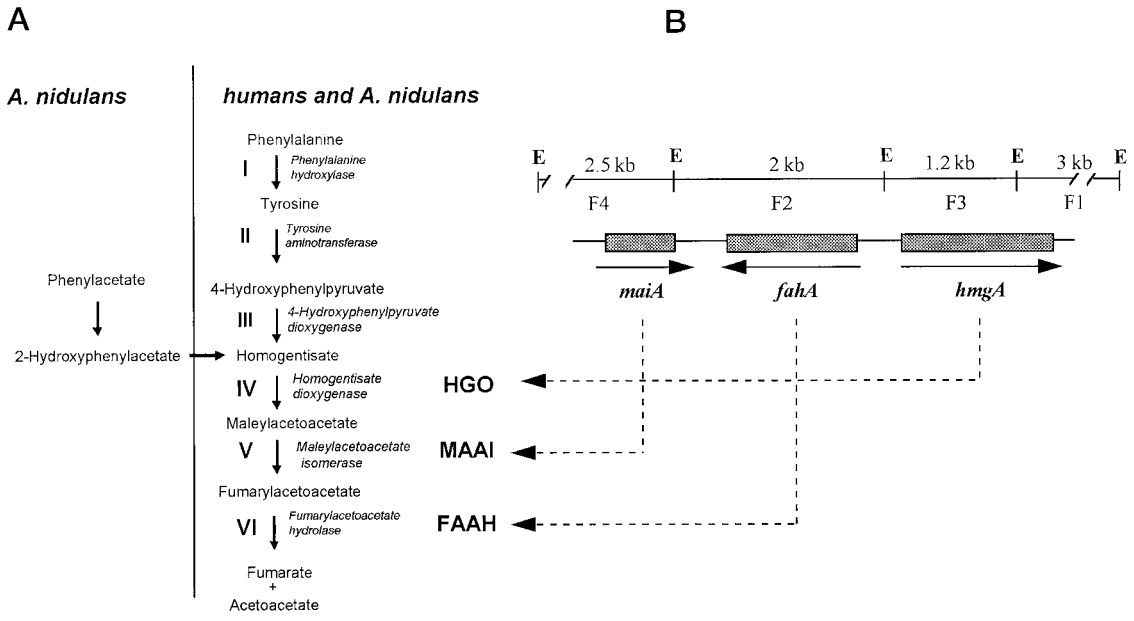

FIG. 1. **The phenylalanine/phenylacetate degradation pathway.** *A*, enzymes required for PhAc degradation to homogentisate are only present in *A. nidulans*. Enzymes for Phe catabolism to fumarate and acetoacetate (1) are present both in humans and *A. nidulans*. Enzymatic steps in the Phe pathway are denoted with roman numbers. The abbreviations for the enzymes used throughout this work are also shown. Single enzyme deficiencies cause the following diseases: *I*, phenylketonuria; *II*, type II (oculocutaneous) tyrosinemia; *III*, type III tyrosinemia; *IV*, alkaptonuria; *V*, (presumably) a variant of type 1 tyrosinemia; *VI*, type 1 tyrosinemia. *B*, the *A. nidulans* cluster for the three genes encoding the common part of the Phe/PhAc degradation pathway. Contiguous genomic DNA fragments (*F1–F4*) and their sizes are indicated. *hmgA* encodes HGO (3), *fahA* encodes FAAH (2), and *maiA* encodes MAAI (this work). *Arrows* indicate the direction of transcription. *E*, *Eco*RI.

DNA Sequencing—Genes and cDNAs were sequenced using a Dye Terminator Cycle sequencing kit (Perkin-Elmer) and *Taq* FS DNA polymerase with universal and custom primers. Sequencing reactions were resolved on an ABI Prism 377 automatic sequencer and analyzed with the ABI analysis software (Version 3.1). Genomic and cDNA versions of *maiA* and human EST cDNA clones 265310 (5′), 290219 (5′), 683733 (5′), and 52677(5′) encoding human MAAI were completely sequenced in both strands. cDNA clones of the IMAGE consortium (25) were purchased from Genome Systems Inc., (St. Louis, MO).[2]

RESULTS

A Cluster of Three Genes Encoding Enzymes of the Common Part of the A. nidulans PhAc/Phe Catabolic Pathway—The ascomycete fungus *A. nidulans* can use either Phe or PhAc as sole carbon source (Fig. 1*A*). Both compounds were catabolized to homogentisate, which was then converted to fumarate and acetoacetate through the action of three enzyme activities, HGO, MAAI, and FAAH. We have previously reported that the *A. nidulans fahA* and *hmgA* genes encoding FAAH and HGO, respectively, are closely linked and divergently transcribed from a 414-base pair intergenic region. *fahA* and *hmgA* gene transcription is strongly inducible by PhAc (or its structural relatives) or Phe and partially repressible by glucose. Neither of these genes is expressed on glucose as the sole carbon source. No gene encoding MAAI has yet been characterized from any organism. Southern blot hybridization of λEMBL4 phage clones carrying the *fahA* and *hmgA* genes with a substracted cDNA probe representing transcripts induced by PhAc revealed the presence of a third linked gene strongly hybridizing to this probe. As *fahA* and *hmgA*, this third gene, designated *maiA*, was not expressed on glucose. Clustering of genes encoding activities of the same catabolic pathway is not unusual in *A. nidulans*. Genomic and cDNA nucleotide sequencing of the region encoding this new transcript confirmed the presence of a third gene 3′ from *fahA*, transcribed in a tail-to-tail orientation (Fig. 1*B*). The transcribed region contains an intron-less ORF encoding a putative 230-residue polypeptide (Fig. 2) whose stop codon is 486 base pairs downstream from that of *fahA*. The nucleotide sequence of this three-gene cluster has been submitted to the DDBJ/EMBL/GenBank™ data bases under accession number AJ001836.

A. nidulans maiA Encodes Maleylacetoacetate Isomerase—Data base searches revealed that the predicted product of *maiA* shows strong amino acid sequence identity to glutathione *S*-transferases. For example, FASTA searches of the Swiss-Prot data base revealed that among the 30 protein sequences showing the highest alignment scores, 27 were glutathione *S*-transferases (data not shown). Similar results were obtained after searching the conceptual translation of EMBL + GenBank™ nucleotide sequence data bases with TBLASTN. The highest FASTA score corresponded to glutathione *S*-transferase 1 from *Diantus caryophyllus*, which showed 33.3% identity to MaiA in a 228-amino acid overlap including the complete sequence of both proteins (data not shown). The deduced molecular mass for MaiA (25,129 Da) is similar to the 25-kDa size of glutathione *S*-transferases.

Northern analysis showed that, as determined for *fahA* and *hmgA*, transcription of *maiA* was induced by either Phe or PhAc and was absent on glucose or gluconeogenic carbon sources (Fig. 3), strongly suggesting that *maiA* was a gene for Phe/PhAc catabolism and that its clustering with *fahA* and *hmgA* reflected its involvement in the same catabolic pathway. The only as yet unidentified gene encoding an enzyme essential for both Phe and PhAc catabolism is that encoding MAAI. The likely mechanism of this enzyme involves transfer of enzyme-bound GSH to C2 of maleylacetoacetate (26). Therefore, all the above data indicated that *maiA* might encode *A. nidulans* MAAI.

To confirm this, we replaced by transformation the wild type *maiA* gene by a mutant version in which the sequence encoding MaiA residues 140–226 had been substituted by a genomic DNA fragment containing the *argB*$^+$ gene (Fig. 4*A*). Transformants were selected in an *argB*2 background for arginine-

[2] Details of EST libraries may be found in http://www-bio.llnl.gov/bbrp/image/humlib_info.html.

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CCGCGGGAATTTGCGCGTCCAGCAGCGGATATTCCATCGCGGAATCCCTTCGGCCACCCC   60

GCATCTATCATTTTTCGAACGTGCTCGCCGCAAAAAGAATCACGTAGCGAAACTAGGAAT  120

GAATAGGAAATCGCCGTGGATACCCTTGGCCAGAGCCTTGAATACGAGCGATTCCGAGTT  180

TATCCTCTAGATAGAGAACTGCATCGATGAACACTTCCAACGCACCACCTTTTCCCTCTA  240

GCAGACTCAAACCGTTTCCAAGCAACAAACCCTAGTCAAGACTGCAATATCTACTTAAAG  300

GGACACCGCACCGGTCAAGATGTCTACAAATTCAGACCTCAGAGTGACACTCTACACCTA  360
                    M  S  T  N  S  D  L  R  V  T  L  Y  T  Y   14

CTTTCGCTCCTCGTGCTCCGCGCGCCTTCGTATCGCCCTCGCCCTTAGGTCAATCTCCTA  420
  F  R  S  S  C  S  A  R  L  R  I  A  L  A  L  R  S  I  S  Y   34

TACCTCAGTCCCTATCAACCTGCTGAAGGGCGAGCAGTCGAGCACAAAAAACACGGCCGT  480
  T  S  V  P  I  N  L  L  K  G  E  Q  S  S  T  K  N  T  A  V   54

AAACCCATCGGCCACCGTTCCCACGCTCATCATCGAGCATGTAGACCGGAGCCAGTCTCC  540
  N  P  S  A  T  V  P  T  L  I  I  E  H  V  D  R  S  Q  S  P   74

AATAACAATAACCCAGTCCCTCGCAGCGCTCGAATACCTGGATGAAGCATTCCCCGACAA  600
  I  T  I  T  Q  S  L  A  A  L  E  Y  L  D  E  A  F  P  D  N   94

TCCCAACCCACTCCTTCCACCTATTTCTAACCCGCAACAACGCGCGCGCTCGTGAGGTCTCT  660
  P  N  P  L  L  P  P  I  S  N  P  Q  Q  R  A  L  V  R  S  L  114

AGCATCAATTATAGCCTGCGACATTCAGCCAGTCACGAACCTACGGATTCTTCAGCGCGT  720
  A  S  I  I  A  C  D  I  Q  P  V  T  N  L  R  I  L  Q  R  V  134

TGCACCATTCGGCGTCGACCGGGCCGCCTGGTCAAAGGATCTCATTGAAGCTGGGTTTGC  780
  A  P  F  G  V  D  R  A  A  W  S  K  D  L  I  E  A  G  F  A  154

GGCGTATGAGGCTATTGCTAGAGACTCTGCTGGAGTATTCAGTGTTGGCGATACAATCAC  840
  A  Y  E  A  I  A  R  D  S  A  G  V  F  S  V  G  D  T  I  T  174

GATGGCGGATGTTTGTTTGATCCCCGCTGTTTGGGGCGCAGAGAGAGCGGGGGTAAATCT  900
  M  A  D  V  C  L  I  P  A  V  W  G  A  E  R  A  G  V  N  L  194

GGGACAGTATCCTACAATTAAAAGGGTTGCGGAGGCCCTGGAGAAGGAGAATGCAGTTAA  960
  G  Q  Y  P  T  I  K  R  V  A  E  A  L  E  K  E  N  A  V  K  214

GGAGGGGCATTGGAGAACCCAGCAGGATACACCAACGGAATTCAGGTGTTGAATCTGCTG 1020
  E  G  H  W  R  T  Q  Q  D  T  P  T  E  F  R  C               230

CTTGGCGACCGATGTTCCATTGTCGGAACGAAAATGTTATACAGGCCGGTCTTGAATGAT 1080

GGACGGGGTTTACTGCGAGAGTAAAAGGTTATCGACAAGTTCGGACAGGAGTCTCTGCGT 1140

ACCCTTGCGCTAAATAAGAGTAATGTAAATGACCTGCAAAGCATCTCCAAAGAAGACCCG 1200

AGTAGCAAATAAAAAAGTCAAAAAAAAGTATGGTCCC                        1237
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FIG. 2. **Nucleotide and deduced amino acid sequence of *A. nidulans maiA*.** The genomic sequence is shown here. The 5′ limit of the cDNA clone showing the longest 5′-UTR is shown with an *arrow*. Sequence downstream from the translation stop codon is shown up to the limit of the cDNA clone carrying the longest 3′-UTR sequence. cDNA sequencing revealed the absence of introns. The sequence has been submitted to DDBJ/EMBL/GenBank™ data bases under accession number AJ001837.

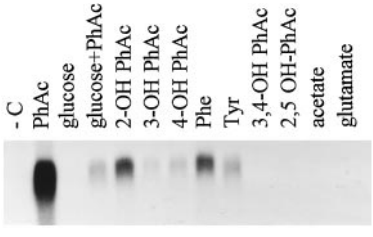


FIG. 3. **Northern analysis of *maiA* transcript levels.** Cells were grown on minimal medium with 0.3% (w/v) glucose as sole carbon source for 16 h at 37 °C and transferred to media with the indicated carbon sources. These secondary cultures were incubated for a further 1 h at 37 °C. Mycelia were then harvested and used to isolate RNA. The probe was a cDNA clone insert containing the complete *maiA* ORF. *-C* indicates no carbon source added.

independent growth and purified by repeated streaking of conidiospores on minimal medium lacking arginine. Two independent transformants showing the expected *maiA* replacement were selected after Southern analysis. Both showed an identical phenotype, being unable to grow on either phenylacetate or phenylalanine as the sole carbon source. This confirmed that *maiA* is a gene of the common part of the Phe/PhAc pathways. MAAI assays were carried out with protein extracts from mycelia of the disrupted strains grown on glucose and transferred to PhAc, which showed them to be deficient for MAAI activity (Fig. 5). By contrast, these extracts showed normal levels of either FAAH or HGO (data not shown). Maleylacetoacetate can be synthesized *in vitro* by the homogentisate dioxygenase activity present in mycelial extracts from the wild type strain or from either a mutant Δ*fahA* strain (lacking FAAH (2)) or a mutant Δ*maiA* strain (presumably lacking MAAI) and detected by its absorption at 330 nm (1, 15, 16). In the absence of GSH, an obligate cofactor of MAAI, maleylacetoacetate is not isomerized to fumarylacetoacetate, thereby providing an enzymatic method to obtain the isomerase substrate (15, 16). On addition of GSH, wild type extracts catalyze the isomerization of maleylacetoacetate to fumarylacetoacetate and the conversion of the latter to fumarate and acetoacetate. As neither of these two latter compounds shows the characteristic absorption of diketoacids in the near ultraviolet region, this coupled enzyme reaction can be monitored by the decrease of A_{330} (see Fig. 5; Refs. 15 and 16). Neither mutant extract alone would catalyze a decrease in A_{330}, as maleylacetoacetate (which would accumulate with the Δ*maiA* extract; see below) and fumarylacetoacetate (which accumu-

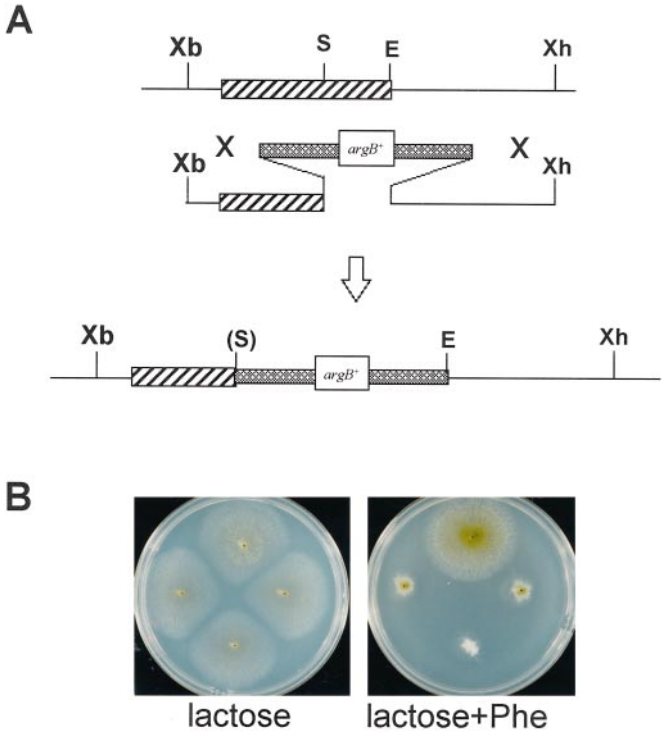
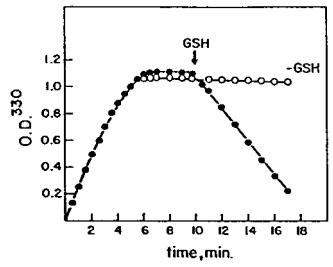


FIG. 4. **Disruption of *maiA*.** *A*, the wild type *maiA* gene was replaced by a mutant version by transformation with a linear *Xba*I-*Xho*I *A. nidulans* genomic DNA fragment in which *maiA* codons 140–226 have been replaced by a 3.2-kb fragment carrying the *argB*$^+$ gene. Transformants carrying the integration event shown were identified by Southern analysis. *Xb*, *Xba*I; *S*, *Sal*I; *E*, *Eco*RI; *Xh*, *Xho*I; *(S)*, a destroyed *Sal*I site. *Dashed box*, the *maiA* coding region; *gray box*, the 3.2-kb *argB*$^+$ fragment. *B*, disruption of *maiA* results in Phe toxicity. Two independent clones (*right* and *left* colonies) carrying the above gene replacement were inoculated on minimal medium with lactose 0.05% (w/v) in the absence (*left panel*) or in the presence (*right panel*) of 25 mM Phe. A wild type (*top*) and a Δ*fahA* strain (*bottom*) were inoculated as controls. Plates were incubated at 37 °C for 96 h.



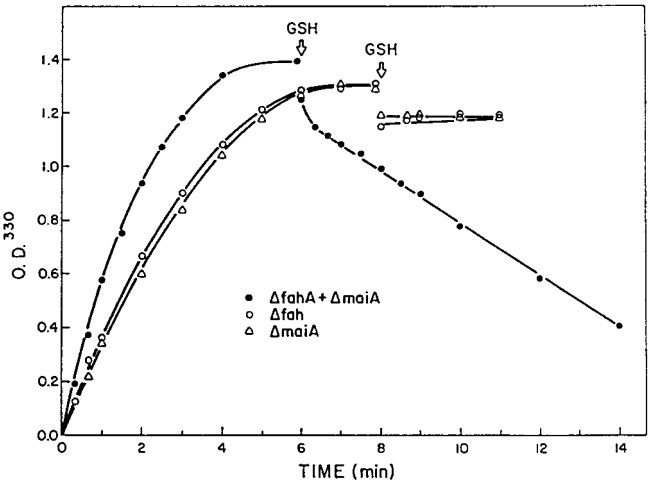


FIG. 5. **Absence of MAAI activity in extracts of the *A. nidulans* strain deleted for the *maiA* gene.** *Large panel*, equal amounts of protein extracts from the indicated mutant *A. nidulans* strains grown under inducing conditions were used either alone or in combination to synthesize maleylacetoacetate from homogentisate. When A_{330} (*O.D.*^{330}) did not increase further, GSH (an obligate cofactor of MAAI) was added at 0.1 mM. A decrease in A_{330} is indicative of maleylacetoacetate conversion to fumarate and acetoacetate through the coupled action of MAAI and FAAH. No decrease indicates that one of these enzyme activities is lacking. The *smaller panel* shows control reactions carried out with a wild type extract. GSH was added at the times indicated with an *arrow*.

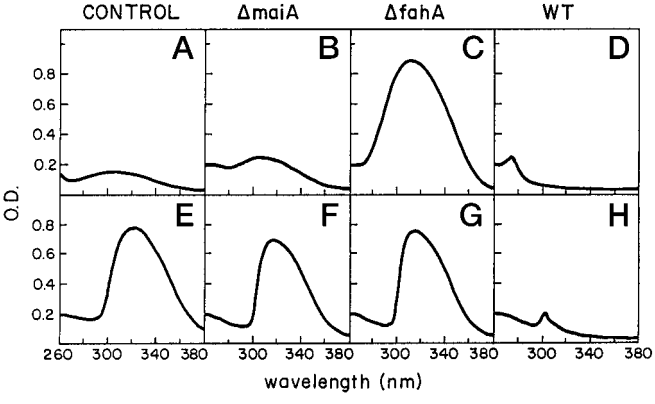


FIG. 6. **Absorption spectra of the compounds accumulated after *in vitro* incubation of maleylacetoacetate with mutant *A. nidulans* protein extracts.** Maleylacetoacetate (*CONTROL* spectra) was synthesized using bacterially expressed HGO and incubated in the presence of equivalent amounts of the indicated protein extracts. Reactions were carried out for 15 min at 23 °C as described under "Experimental Procedures." *A–D*, acid pH; *E–H*, neutral pH; *O.D.*, absorbance.

lates with the Δ*fahA* extract) have similar ϵ_{330} at neutral pH (15). By contrast, a mixture of the two mutant extracts should provide both the isomerase and the hydrolase, and their coupled action should result in conversion of maleylacetoacetate to fumarate and acetoacetate. Fig. 5 shows that these predictions were fulfilled by mutant extracts. We concluded that the Δ*fahA* extract provided an activity required for catabolism of maleylacetoacetate that was missing in the Δ*maiA* extract. Sequential addition of extracts instead of their simultaneous mixing resulted in substantially identical results (data not shown). Taken in the context of the enzymes known to be required for homogentisate degradation (Fig. 1*A*), these data further indicated that MaiA is a fungal MAAI.

In contrast to fumarylacetoacetate, which shows a similar ϵ_{330} at both pH 1 and pH 7.5, maleylacetoacetate shows a 10-fold lower ϵ_{330} at pH 1 than at pH 7.5 (15). Therefore, a fall in A_{330} upon acidification of the homogentisate dioxygenase reaction product is diagnostic of maleylacetoacetate (Fig. 6, *A* and *B*; it should be noted that the absorption maximum for the above compounds is at approximately 320 nm (see also Ref. 15). This was used to confirm its accumulation with the Δ*maiA* but not with the Δ*fahA* extract. Maleylacetoacetate was prepared using recombinant human HGO expressed in *E. coli* as described under "Experimental Procedures" and incubated in the presence of the wild type or mutant extracts and GSH. Incubation with the wild type extract resulted in the disappearance of compounds absorbing in the near ultraviolet region at either acidic or neutral pH (Fig. 6, *D* and *H*). By contrast, the characteristic absorption of maleylacetoacetate at neutral but not at acidic pH was still detectable upon incubation with the Δ*maiA* extract (Fig. 6, *B* and *F*). Incubation of the substrate with the Δ*fahA* extract (which contains the isomerase but not the hydrolase) produced a compound (fumarylacetoacetate) showing strong absorbance in the above region both at acidic and neutral pH (Fig. 6, *C* and *G*).

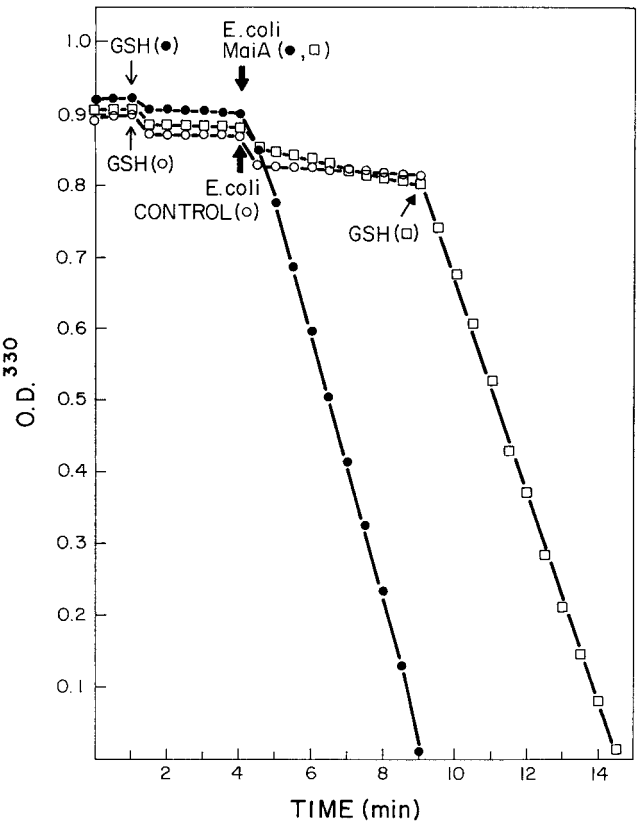


FIG. 7. **MAAI activity of *A. nidulans* MaiA synthesized in *E. coli*.** An *A. nidulans* Δ*maiA* extract (lacking MAAI, see Figs. 5 and 6) was used in three identical, separate reactions to synthesize maleylacetoacetate, which was monitored by its A_{330} ($O.D.^{330}$). When A_{330} showed no further increase, GSH (150 μM, *small arrows*) and protein extracts (50 μg, *large arrows*) from control *E. coli* cells or from *E. coli* cells overexpressing MaiA were added as indicated. Degradation of maleylacetoacetate to fumarate and acetoacetate was monitored by the decrease of A_{330} and required the addition of both bacterially expressed MaiA and GSH.

Expression of A. nidulans MaiA in E. coli—To definitively establish that MaiA is *A. nidulans* MAAI, we expressed the polypeptide as a fusion protein (see "Experimental Procedures") in *E. coli* under the control of a T7 RNA polymerase-dependent promoter. Promoter induction resulted in the synthesis of a markedly abundant 32-kDa protein that was absent from extracts of induced cells carrying the expression vector with no insert. This protein, whose electrophoretic mobility was roughly consistent with the M_r predicted for the MaiA fusion protein, remained soluble upon clarification of the cell lysate (data not shown). *In vitro* complementation of *A. nidulans* Δ*maiA* and Δ*fahA* protein extracts described above suggested the use of Δ*maiA* extracts in a coupled enzyme assay for MAAI. Δ*maiA* extracts are unable to transform homogentisate to fumarate and acetoacetate unless supplemented with MAAI enzyme. Fig. 7 shows that an extract from *E. coli* cells overexpressing MaiA complemented this MAAI deficiency *in vitro.* The coupled action of bacterially expressed MAAI and endogenous *A. nidulans* FAAH resulted in the efficient degradation of maleylacetoacetate. This coupled degradation obligately required GSH and did not occur with an extract of *E. coli* cells carrying the expression vector with no insert (Fig. 7).

In addition, we synthesized maleylacetoacetate using recombinant human homogentisate dioxygenase. It showed the diagnostic high absorbance in the near ultraviolet region under neutral but not under acidic pH conditions (see above). Incubation of this compound in the presence of the *E. coli* extract overexpressing MaiA and GSH resulted in its conversion to fumarylacetoacetate, as shown by the characteristic high absorbance of the latter in this region both under acidic and neutral pH conditions. This conversion did not take place when extracts overexpressing the protein were substituted by control extracts (data not shown). As a whole, these results provide formal evidence that *A. nidulans maiA* encodes MAAI.

Analysis of A. nidulans Culture Supernatants for Diagnostic Compounds of MAAI Deficiency—An *A. nidulans* strain disrupted for the gene encoding FAAH accumulates succinylacetone, the diagnostic compound for HT1 (caused by FAAH deficiency) in the urine of human patients. Growth of a Δ*fahA* strain on lactose (which is unaffected by the mutation) is strongly inhibited by Phe ((2); see Fig. 4*B*). Phe toxicity in this mutant background is due to fumarylacetoacetate and/or its spontaneous reaction product, succinylacetone, which accumulate(s) as a result of the enzyme deficiency. Notably, growth of a strain disrupted for *maiA* was also inhibited by Phe (Fig. 4*B*), although clearly to a lesser extent than that of the Δ*fahA* strain, strongly suggesting that different catabolites with distinct toxicities accumulate in each disrupted strain (but see "Discussion").

No specific diagnostic method is available for a deficiency in human MAAI. We therefore investigated by GC-MS the compounds accumulated in a culture supernatant of our Δ*maiA* strain transferred to phenylacetate and detected four peaks almost certainly corresponding to *cis* and/or *trans* isomers of 4,6-dioxohept-2-enoic acid (Fig. 8), as shown by comparison of their mass spectra with those for the above compound(s) deposited in the data bases. These four peaks presumably represent different TMS derivatives of the above compound(s) (see legend to Fig. 8). Neither of the two possible *cis-trans* isomers of 4,6-dioxohept-2-enoic acid, maleylacetone and fumarylacetone, had been detected in culture supernatants of either the wild type or a Δ*fahA* strain (2), strongly suggesting that its (their) presence specifically results from the Δ*maiA* mutation. Notably, no peaks corresponding to succinylacetone were detected.

Identification of Human EST Clones Encoding Homologues of A. nidulans MaiA—We next used the fungal MaiA amino acid sequence to screen the EST data bases for human and murine ESTs encoding putative MaiA homologues, in analogy with the protocol already established for the *AKU* gene (3, 4). BLAST searches identified a number of these human ESTs. The 10 highest scores were obtained with the derived amino acid sequences of the following human ESTs (with the source of RNA for each EST cDNA clone in parentheses): 683733 (germinal B cells), 290219 (multiple sclerosis), 290775 and 265310 (melanocytes), 52677 (infant brain), 156401 (breast), 309975 (senescent fibroblasts), 240726 and 246479 (fetal liver/spleen), and 66e04 (skeletal muscle). These (partial) amino acid sequences showed more than 40% identity to that of *A. nidulans* MaiA, strongly suggesting that they represent its human homologue(s). The fact that only minor differences in the sequence (presumably resulting from automated sequencing errors) were found between these derived human proteins strongly suggests that all these cDNAs correspond to a single gene. Tissue-specific expression of such a gene does not appear to be as restricted as that of HGO (3, 4), and only two of the 10 cDNA clones represented liver transcripts (see above). In addition to the above human protein sequences, data base searches detected MaiA homology to derived protein products of mouse and *Arabidopsis thaliana* EST clones (not shown).

Molecular Characterization of Human cDNAs Encoding Maleylacetoacetate Isomerase—We fully sequenced four of the above ESTs. (Fig. 9*A*). Nucleotide sequencing showed that they were indeed encoded by the same gene, despite the fact that

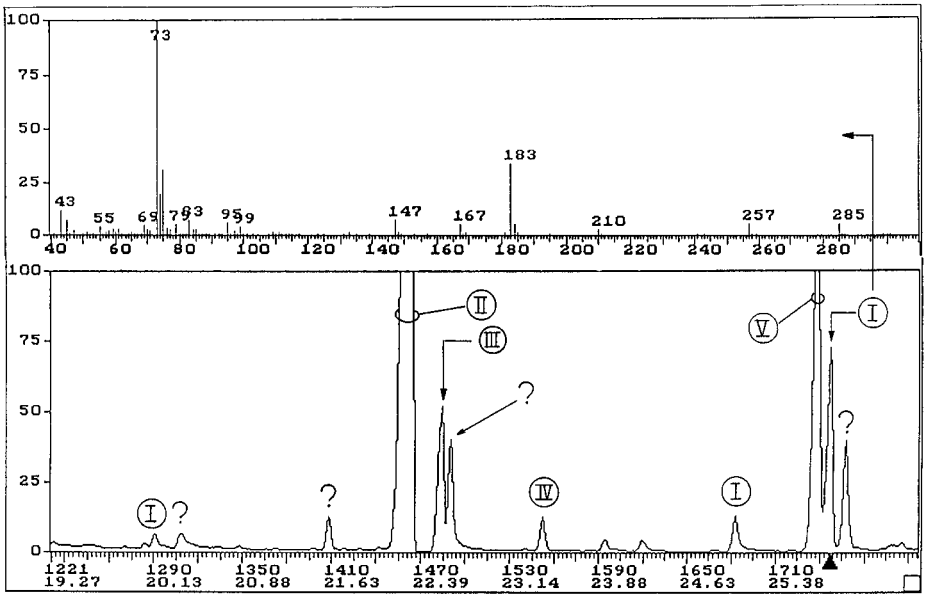
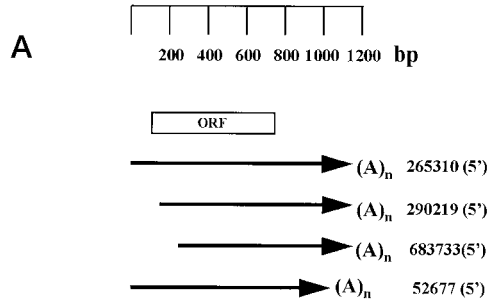


FIG. 8. **GC-MS analysis of a culture supernatant of the Δ*maiA* strain.** Ether-extractable compounds were analyzed as TMS derivatives by GC-MS. Identified peaks were assigned to a compound by computer comparison of their mass spectra with reference spectra of the NIST/EPA/NIH mass spectra data base. A selected portion of the chromatogram including three peaks (denoted with *roman number I*) that were identified as di-TMS derivatives of 4,6-dioxohept-2-enoic acid is shown at the bottom. Peaks labeled with *?* represent unidentified compounds. *Peak II*, 2-hydroxyphenylacetate; *III*, ethylvanilline (internal standard, ~0.2 μg); *IV*, 3-hydroxyphenylacetate; *V*, possibly octadecenol. The mass spectra of the major peak of 4,6-dioxohept-2-enoic acid (di-TMS) (indicated with a *filled arrowhead* in the chromatogram) is shown. A fourth peak (not shown in this portion of the chromatogram) was identified as the tri-TMS derivative of 4,6-dioxohept-2-enoic acid.



B

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                                          (AN) MSTNSDL   7

                                          (HS) MQAG      4

(AN)    8 RVTLYTYFRSSCSARLRIALALRSISYTSVPINLLK..GEQSSTKNTAVN 55
            || |||||||  | ||||||  |  |  |||||  |  | | |    | |
(HS)    5 KPILYSYFRSSCSWRVRIALALKGIDYKTVPINLIKDGGQQFSKDFQALN 54

(AN)   56 PSATVPTLIIEHVDRSQSPITITQSLAALEYLDEAFPDNPNPLLPPISNP 105
          |   ||||  |          |||  ||||  |||  |  |      |||    |
(HS)   55 PMKQVPTLKID.......GITIHQSLAIIEYLEETRP..TPRLLPQ..DP 93

(AN)  106 QQRALVRSLASIIACDIQPVTNLRILQRVAPFGVDRAAWSKDLIEAGFAA 155
            || ||      ||  |||  ||  |  |          |    |  || |
(HS)   94 KKRASVRMISDLIAGGIQPLQNLSVLKQVG..EEMQLTWAQNAITCGFNA 141

(AN)  156 YEAIARDSAGVFSVGDTITMADVCLIPAVWGAERAGVNLGQYPTIKRVAE 205
            | |    ||   |||  ||||  || | |  |||   | |  ||||
(HS)  142 LEQILQSTAGIYCVGDEVTMADLCLVPQVANAERFKVDLTPYPTISSINK 191

(AN)  206 ALEKENAVKEGHWRTQQDTPTEFRC 230
            |    |    |   | |||||  |
(HS)  192 RLLVLEAFQVSHPCRQPDTPTELRA 216
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FIG. 9. **Characterization of human MAAI cDNAs and derived amino acid sequence of human MAAI.** *A*, four EST cDNAs encoding putative MaiA homologues were fully sequenced. The corresponding four mRNAs appear to be transcribed from the same gene (see text for details). The limits of these four cDNAs with respect to the human MAAI coding region are shown. *B*, amino acid sequence of human MAAI (*HS*) and its comparison to its fungal homologue (*AN*). The amino acid sequence of the 216-residue-derived protein encoded by the EST 265310 (5′) ORF (and the three other EST ORFs above) is shown compared with that of *A. nidulans* MaiA (this work). *Vertical bars* indicate identity. The alignment was carried out using BESTFIT (GCG package).

they had been isolated from different tissues. EST 265310 (5′) (melanocytes) is the longest of these cDNAs. It is 1155 base pairs long (excluding the poly(A) tail) and contains a 216-codon ORF whose derived protein product (M_r 24,083) shows 45% identity in amino acid sequence to *A. nidulans* MAAI (Fig. 9*B*). This represents nearly definitive evidence that this cDNA encodes a human MAAI (but see below). The complete nucleotide sequence of this cDNA has been submitted to DDBJ/EMBL/GenBank™ data bases (accession number AJ001838) The 3′-UTR of this transcript was remarkably long (400 nucleotides, *i.e.* 30% of the transcript size). ESTs 290219 (5′) (multiple sclerosis lesions) and 683733 (5′) (germinal B-cells) represented cDNAs incomplete at their 5′ ends, starting at codons 18 and 49, respectively, of the human MAAI ORF. The precise site of polyadenylation and the sequence of the 400-nucleotide 3′-UTR of EST 290219 were identical to those of the longest cDNA. Polyadenylation of the 683733 cDNA occurred two nucleotides upstream of the above site, but no other nucleotide sequence difference was observed either in the 3′-UTR or in the coding region. Finally, we detected no differences between the nucleotide sequences of EST 52677 (brain) and EST 265310 up to position 1045, where the former is prematurely polyadenylated as compared with the latter. This strongly suggests that the 5′ ends of these two transcripts represent a transcription start site for the human MAAI gene.

To confirm that the ORF of these transcripts encodes a human MAAI, the protein product encoded by the EST 265310 (5′) ORF was overexpressed in *E. coli* as above. High levels of a protein with the expected mobility for this polypeptide product were detected by SDS-polyacrylamide gel electrophoresis when recombinant *E. coli* cells in which its expression was driven by a T7-polymerase promoter (see "Experimental Procedures") were grown under inducing conditions (data not shown) and were absent from these cells grown under noninducing conditions. Extracts from *E. coli* cells overexpressing this protein showed strong maleylacetoacetate isomerase activity, as shown by its ability to complement an *A. nidulans* extract deficient for the enzyme (Fig. 10). This strong MAAI activity was dependent on the presence of GSH (not shown). By contrast, control cells showed no MAAI activity. These results establish that the product of the EST 265310 ORF (and, by extension, of the above four cDNAs) is a human maleylacetoacetate isomerase.

DISCUSSION

We describe here the molecular characterization of *maiA*, a gene encoding an enzyme of the common part of the *A. nidulans*

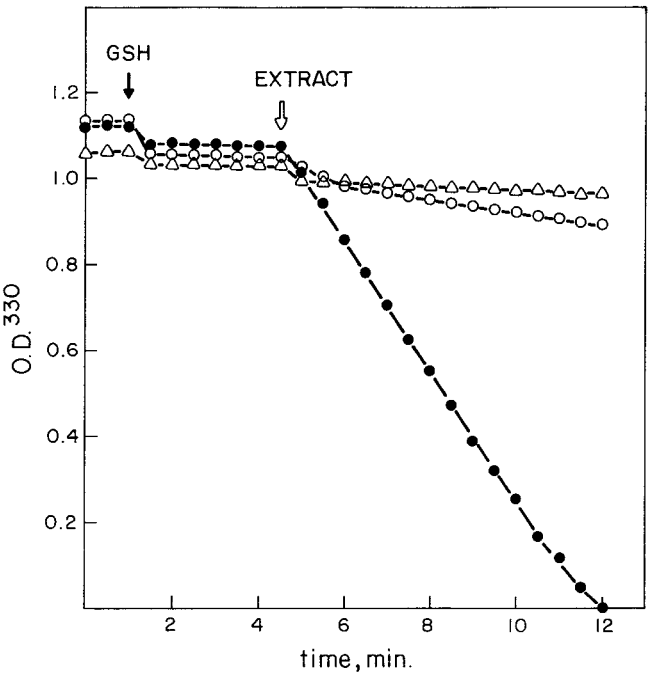

FIG. 10. **The product of the human EST 265310 (5′) ORF has MAAI activity.** Detection of MAAI activity in cleared lysates of *E. coli* cells that overexpressed the protein encoded by this ORF under the control of a T7 polymerase-dependent promoter (see "Experimental Procedures"). The assay for MAAI, based on complementation of an *A. nidulans* Δ*maiA* extract, was as in Fig. 7. The time points at which reduced glutathione (150 μM) and protein extracts (~50 μg) were added is indicated. *Filled circles*, a reaction to which a bacterial extract overexpressing the product of the EST 265310 ORF was added. The levels of expression achieved for this protein (not shown) were similar to those for *A. nidulans* MaiA. *Triangles*, an extract from induced *E. coli* cells carrying the expression vector with no insert. *Open circles*, a second, negative control reaction in which an extract from a recombinant bacterial clone that showed no overexpression of the desired EST 265310 protein (as determined by SDS-polyacrylamide gel electrophoresis) was added. $O.D.^{330}$, A^{330}.

phenylalanine/phenylacetate pathways and provide definitive biochemical and genetic evidence that this gene encodes a maleylacetoacetate isomerase. This paper represents the first characterization of a gene encoding MAAI from any organism. Compelling evidence for the above conclusion can be summarized as follows: (i) *maiA* is clustered with *fahA* and *hmgA*, the two other structural genes of this common part of the pathways; (ii) transcription of the gene is induced by phenylalanine or phenylacetate as sole carbon sources; (iii) the deduced amino acid sequence of its encoded protein shows identity to glutathione *S*-transferases, as expected for a MAAI enzyme; (iv) disruption of the gene prevents growth on either Phe or phenylacetate; (v) protein extracts from this disrupted strain convert homogentisate to maleylacetoacetate but cannot catabolize this compound further; (vi) mixing Δ*maiA* and Δ*fahA* extracts results in reciprocal complementation of the corresponding enzyme deficiencies required for maleylacetoacetate catabolism to fumarate and acetoacetate; (vi) expression of *maiA* in *E. coli* results in bacterial protein extracts showing MAAI activity. Two technical developments were crucial to obtaining some of the above evidence. First, we used either recombinant HGO enzyme (4) or fungal extracts showing high HGO activity (21) to efficiently synthesize maleylacetoacetate. Second, we used a complementation assay for MAAI based on a protein extract from our *A. nidulans* strain deleted for *maiA*. This extract converted homogentisate to fumarate and acetoacetate only when supplied with GSH and a source of MAAI.

We next used the MaiA-derived sequence to identify human, mouse, and plant ESTs encoding proteins showing high amino acid sequence identity to *A. nidulans* MAAI. Four such human cDNAs were fully sequenced and shown to encode a protein with 45% identity to MaiA. Although they were isolated from different tissues, these four cDNAs (and the other ESTs detected in our searches) almost certainly represent transcripts of the same gene. The protein encoded by this transcript(s) has MAAI activity when expressed in *E. coli*. Our electronic screening of the human EST data base would suggest that expression of this gene would be more ubiquitous than that of HGO, whose expression is largely restricted to liver, kidney, colon, small intestine, and prostate. This apparently less restricted pattern of expression might be related to its ability to use other compounds, in addition to maleylacetoacetate, as substrates (26), which might suggest a detoxification function (6).

Our characterization of human MAAI cDNAs represents the identification of the only as yet undescribed gene of the human Phe catabolic pathway. These results further confirm the validity of our fungal metabolic model and open the possibility of analyzing at the molecular level the predicted disease (a possible variant of HT1) resulting from MAAI deficiency. The incidence of this inborn error of metabolism is presently unknown, perhaps due to the absence of clear biochemical and/or molecular diagnostic criteria. A single patient with a putative MAAI deficiency has been reported in an abstract (14). Notably, this patient suffered from severe hepatorenal and brain damage. Our results with the fungal model show that the metabolite(s) accumulated as a result of a MAAI deficiency is indeed toxic for *Aspergillus*, but their toxicity is detectably lower than the toxicity of those accumulated as a result of a FAAH deficiency. Jorquera and Tanguay (12) have reported that, in contrast to fumarylacetoacetate, maleylacetoacetate was not mutagenic in Chinese hamster cells. We have not yet addressed if either of the above deficiencies is mutagenic in *Aspergillus*.

We detected no succinylacetone in culture supernatants of the *A. nidulans* Δ*maiA* strain. This would be expected from the presumed origin of succinylacetone from decarboxylation of succinylacetoacetate, as normal levels of FAAH in this strain would degrade the latter (1). Therefore, this absence of succinylacetone might, at least in part, account for the lower Phe toxicity found in an *A. nidulans* strain deficient for MAAI as compared with a strain deficient for FAAH. Analysis of culture filtrates of the *A. nidulans* Δ*maiA* strain specifically detected the presence of 4,6-dioxohept-2-enoic acid. This chemical structure would be consistent with maleylacetone (*cis* isomer) and/or fumarylacetone (*trans* isomer). These isomers cannot be reliably distinguished by the methodology used here. Taking into account the remarkable similarities in the consequences of equivalent metabolic blocks in human and fungal Phe catabolism, we suggest *cis* and/or *trans* isomers of 4,6-dioxohept-2-enoic acid as possible diagnostic compound(s) for MAAI deficiency in humans.

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Note Added in Proof—An unpublished sequence recently submitted to the GenBank™/EBI Data Bank with accession number U86529 and described as a cDNA encoding a human glutathione transferase Zeta 1 is the same as our cDNA sequence for human maleylacetoacetate isomerase.

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Characterization of a Fungal Maleylacetoacetate Isomerase Gene and Identification of Its Human Homologue

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