Aspergillus nidulans is able to use Leu as the sole carbon source through a metabolic pathway leading to acetyl-CoA and acetoacetate that is homologous to that used by humans. meca and mecB, the genes encoding the subunits of 3-methylcrotonyl-CoA carboxylase, are clustered with ivdA encoding isovaleryl-CoA dehydrogenase, a third gene of the Leu catabolic pathway, on the left arm of chromosome III. Their transcription is induced by Leu and other hydrophobic amino acids and repressed by glucose. Phenotypically indistinguishable Δmeca, ΔmecB, and Δmeca ΔmecB mutations prevent growth on Leu but not on lactose or other amino acids, formally demonstrating in vivo the specific involvement of 3-methylcrotonyl-CoA carboxylase in Leu catabolism. Growth of mec mutants on lactose plus Leu is impaired, indicating that Leu metabolite(s) accumulation resulting from the metabolic block is toxic. Human patients carrying loss-of-function mutations in the genes encoding the subunits of 3-methylcrotonyl-CoA carboxylase suffer from methylcrotonylglycinuria. Gas chromatography/mass spectrometry analysis of culture supernatants revealed that fungal Δmec strains accumulate 3-hydroxyisovaleric acid, one of the diagnostic compounds in the urine of these patients, illustrating the remarkably similar consequences of equivalent genetic errors of metabolism in fungi and humans. We use our fungal model(s) for methylcrotonylglycinuria to show accumulation of 3-hydroxyisovalerate on transfer of 3-methylcrotonyl-CoA carboxylase-deficient strains to the isoprenoid precursors acetate, 3-hydroxy-3-methylglutarate, or mevalonate. This represents the first report of metabolic steps in this pathway is catalyzed by the branched chain amino acid (BCAA) transaminase, which appears to act on all three BCAAs (Leu, Ile, and Val) to yield the corresponding branched chain ketoacids (BCKAs). All three ketoacids are substrates of the BCKA dehydrogenase complex, which catalyzes their oxidative decarboxylation to the respective 1 acyl-CoA derivatives (Fig. 1, step II, isovaleryl-CoA in the case of Leu catabolism). Isovaleryl-CoA is oxidized to acetyl-CoA and acetoacetate through four additional steps specific for Leu catabolism (Fig. 1, steps III–VI) (2).

Individuals deficient for any of the six enzymes of this pathway have a clinical phenotype (see Fig. 1). For example, inborn errors of metabolism resulting from loss-of-function mutations in any of the structural genes encoding subunits of the BCKA dehydrogenase complex lead to maple syrup urine disease, a severe, not infrequent panethnic recessive disorder (2). However, despite the clinical relevance of this pathway, the molecular basis of two enzyme deficiencies in Leu catabolism had not been established when this work was started. These were the deficiencies of 3-methylcrotonyl-CoA carboxylase (MCC; EC 6.4.1.4), resulting in 3-methylcrotonylglycinuria (OMIM 210200), and 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18), resulting in type I 3-methylglutaconic aciduria (OMIM 250950). Whereas the characterization of the 3-methylglutaconyl-CoA hydratase-mediated step will be the subject of another paper, this paper describes the genetic and biochemical characterization of the MCC-mediated step in Aspergillus nidulans. The derived amino acid sequences of the fungal MCC subunits were instrumental for our identification of their human homologues and subsequent characterization of the molecular basis of the disease (3), which was simultaneously reported by Baumgartner et al. (4).

The mitochondrial enzyme MCC is, with propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), and acetyl-CoA carboxylase (ACC), one of the four biotin-dependent carboxylases in humans. All four enzymes share a biotin carrier domain carrying the covalently bound prosthetic group, a biotin-carboxylating domain, and a carboxyltransferase domain catalyzing carboxyl group transfer from carboxybiotin to the specific substrate. In the current model (5), the biotin carrier domain acts as a swinging arm oscillating between the biotin carboxylase and the carboxyltransferase domains of the enzyme. As PCC, MCC is a heterodimeric enzyme consisting of α and β subunits in an (αβ)n configuration (6), whose correspond-
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Fig. 1. The leucine degradation pathway and the mevalonate shunt. Shown are enzymes for Leu catabolism in Aspergillus and humans, with other possible pathways involving 3-methylcrotonyl-CoA carboxylase. Enzyme deficiencies in Leu catabolism cause the following diseases: hypervalinemia/hyperleucine-isoleucinemia (I); maple syrup urine disease (II); isovaleric academia (III); isolated 3-methylcrotonyl-CoA carboxylase deficiency, methylcrotonylglycinuria (IV); methylglutacemic aciduria, type 1 (V); and 3-hydroxy-3-methylglutacemic aciduria (VI). The isoprenoid biosynthetic pathway is schematized on the right, with an indication of proposed intermediates of the mevalonate shunt (see "Discussion"). Geranyol-CoA degradation could be another possible link between isoprenoid metabolism and the Leu degradation pathway.

Experimental Procedures

Fungal Strains, Media, and Growth Conditions—A. nidulans strains carried markers in standard use (15). Complete and minimal media were as in Ref. 16. Lactose was used at 0.05% (w/v) as the sole carbon source, and amino acids were used at 30 μM, unless otherwise indicated.

RNA Isolation—7 × 10⁶ conidia/ml were inoculated on minimal medium containing 0.3% (w/v) glucose as the sole carbon source and 5 mM ammonium tartrate as nitrogen source. Primary cultures were incubated at 37 °C. When the glucose concentration fell below 0.01%, mycelia were transferred to 5 mM urea minimal medium without any carbon source and incubated for a further 1 h before adding an appropriate carbon source. Secondary cultures were incubated for a further 3 h before collecting mycelia for RNA extraction. Mycelia were frozen in liquid nitrogen; ground to fine powder; and resuspended in 6 M...
guanidine hydrochloride, 0.1 mM dithiothreitol, and 30 mM sodium acetate, pH 5.0, and incubated for 10 min at 0 °C. This suspension was spun at 10,000 rpm and 4 °C for 10 min in a SS-34 rotor, and nucleic acids were resuspended in 20 mM EDTA, pH 8.0, and the RNA was precipitated with 3 volumes of 4 mM sodium acetate at 1 h at −20 °C and recovered after centrifugation in a refrigerated microcentrifuge. RNA was resuspended in RNAase-free water, reprecipitated with ethanol, and stored in water at −80 °C.

Probes for Genomic Library Screens and Northern Blot Analysis—The screens of the genomic library were carried out using PCR-amplified DNA fragments obtained from genomic DNA (mccA) or from A. nidulans ESTs n01c10 (mccB) (available on the World Wide Web at bioinfo.okstate.edu/pipeonline/). In Northern blots, the EST n01c10 insert was also used as an mccB-specific probe, the mccA probe was a PCR fragment containing the complete mccA coding sequence, and the idaA-specific probe included its coding region between codon 69 and the stop codon.

Genomic and cDNA Libraries—The A. nidulans genomic library was a standard AEBLBA library constructed from a Glasgow wild-type strain. mRNA used to construct the cDNA library was obtained from mycelia cultured in the presence of 30 mM Leu as the sole carbon source, which strongly induces transcription of Leu catabolism genes. After oligo(dT) selection of messenger RNA, double-stranded cDNA was synthesized using the SMART™ technique (Clontech), ligated into SfiI-digested XpliP32 phagemid arms, and packed in vitro using Stratagene Gigapack II Plus extracts. The primary library contained 1.5 × 106 recombinant clones, with an average insert size of 1.39 kb.

Transformation—To construct ΔmccA and ΔmccB mutants, strains MAD003 bij1a mgh1 argB2 and MAD559 yA2 paba1 argB2 pyrG89 riboB2 were used as recipient strains in transformation experiments (15). One ml of the transformation mixture containing mccA- and mccB-specific probes or mccB- and pyr-de-specific probes was used to confirm the expected deletion events in ΔmccA and ΔmccB strains, respectively.

Enzyme Assays—These were carried out essentially as described (15), by determining the amount of [14C]HCO3− incorporated by protein extracts into acid-soluble material in a 3-methylcrotonyl-CoA- and ATP-dependent reaction mixture. The reaction mixture contained, in a final volume of 0.2 ml, 0.1 mM Tris-HCl, pH 8.0, 0.1 mM KCl, 6 mM MgCl2, 1.5 mM dithiothreitol, 1 mM ATP, and 6 mM [14C]HCO3− (16 μCi/μmol). Reactions were started by the addition of 300 μg 3-methylcrotonyl-CoA. After a 7-min incubation at 30 °C, they were stopped by the addition of 0.1 ml of 6 N HCl. Samples were microcentrifuged for 15 min at 14,000 rpm, and aliquots of the supernatant were spotted on Whatman 3MM paper, dried, and counted in a liquid scintillation counter.

GC/MS Analysis of Culture Supernatants—Fungal mycelia were precultured as described for RNA isolation and transferred to minimal medium with one of the following carbon sources: 0.05% (v/v) lactose, 3 mM Leu, 10 mM Ile, 100 mM sodium acetate, 30 mM 3-hydroxy-3-methylglutaric acid, or 30 mM pimelate. Secondary cultures were incubated for a further 24 h. Culture supernatants were collected after removing mycelia by filtration. After the addition of 0.1 ml (final concentration) phenylacetate (which was used as an internal control for extraction of organic acids), culture supernatants were acidified to pH <2 with 6 N HCl in the presence of saturating NaCl and extracted with isobutylacetate. The organic phase was dried under nitrogen and derivatized with bis(trimethylsilyl)trifluoroacetamide. Trimethylsilyl derivatives were analyzed in a 5% phenylmethylsilicone column (30 m × 0.25 mm; 0.25-μm film thickness), using an HP 5890 Series II gas chromatograph and an HP 5971 mass spectrometer, with the following temperature program. After 5 min at 70 °C, the temperature was raised to 300 °C (4.5 °C/min) and kept at this limit for a further 5 min. Identification of peaks was carried out with the ChemStation software of Hewlett Packard.

RESULTS

Previous work showed that Leu is able to support A. nidulans vegetative growth and conidiation when used as the sole carbon source, although both growth strength and conidiation density were notably reduced on a range of Leu concentrations as compared with more favorable carbon sources. Supplementation of the medium with pyridoxine or thiamine (the co-factors of BCAA transaminase and BCKA dehydrogenase, respectively) did not improve either to any significant extent, indicating that co-factor biosynthesis is not limiting. Thus, unless otherwise indicated (see GC/MS analysis below), we routinely used Leu at 30 mM for growth tests and induction of the synthesis of Leu catabolic enzymes.

Molecular Characterization of Fungal Genes Encoding Subunits of 3-Methylcrotonyl-CoA Carboxylase—Fungal candidate cDNAs for MCCa and MCCβ were identified by in silico screening of the University of Oklahoma A. nidulans EST database (available on the World Wide Web at www.genome.ou.edu/fungal.html) using the deduced Arabidopsis thaliana a- and β-MCC and human α- and β-PCC amino acid sequences in TBLASTX searches (3). PCR-derived DNA probes, based on the nucleotide sequences of candidate ESTs, were obtained and used to screen A. nidulans genomic (in AEMBLA) and cDNA (from inducing growth conditions; see below) libraries. The genomic screen revealed, in addition to phage clones hybridizing only to MCCa- or MCCβ-specific probes, a third class of clones cross-hybridizing to both, which strongly suggested that A. nidulans candidate genes encoding MCCa and MCCβ, which we denoted mccA and mccB, respectively, are physically linked. Restriction enzyme mapping and nucleotide sequencing of an −15-kb A. nidulans genomic DNA region reconstructed with overlapping bacteriophage clone inserts in this third class revealed that this is indeed the case. mccA and mccB open reading frames are divergent, with their predicted initiation Met codons separated by 3692 bp.

A cDNA library made from mRNA isolated from cells grown in the presence of Leu as the sole carbon source (a condition inducing transcription of Leu catabolic genes; see below) was screened to isolate full-length mccA and mccB cDNAs, which were used to determine intron/exon borders after comparison of genomic and cDNA sequences. The 2688-bp mccA gene (GenBank™ accession number AY387592) is split by three introns ranging from 44 to 309 bp, whereas the 1843-bp mccB gene (GenBank™ accession number AY387593) is split by a single 80-bp intron.

The mccA cDNA encodes a 763-amino MccA polypeptide that shows 40% amino acid sequence identity to Arabidopsis and human MCCα. Triple (fungal, plant, and human) MCCα alignment (not shown) showed that patches of amino acid sequence conservation predominate in the NH2-terminal two-thirds of the protein and in the −65-residue C-terminal domain containing the biotinylation motif, whereas the sequence separating these regions (residues 480–640 in A. nidulans MccA) does not contribute to sequence conservation. Near the C terminus of MccA there is a 666 SMKMKtetrapeptide biotin binding motif found in biotin-dependent carboxylases (19), where the ϵ-amino group of Lys-670 is predicted to bind the co-factor covalently through an amide bond. In addition, A. nidulans MccA contains the sequence 197GGGGKKMR204, which is completely conserved among Aspergillus, Arabidopsis, and human MCCαpolypeptides and strongly conserved among biotin-dependent carboxylases. The structure of bacterial acetyl-CoA carboxylase revealed that this peptide makes a major contribution to ATP binding (20).

The mccB cDNA encodes a 567-residue MccB polypeptide showing 59 and 51% amino acid sequence identity to human and Arabidopsis MCCβ, respectively. MccB contains a putative acyl-CoA binding motif (21, 22) within residues 354 and 392, which is strongly conserved among MCCβ subunits and less so among biotin-dependent carboxylases, possibly reflecting the differing substrate specificities of these enzymes. In agreement with substrate specificities residing in the β chains of heterodimeric biotin-dependent carboxylases, MccB and PCCβ are markedly more dissimilar in amino acid sequence than the MccA/PCCα pair (data not shown).

Transcriptional Regulation of mccA and mccB—Northern analysis of mycelia precultured under glucose-limiting condi-
catabolism. Centromere distal from \( mcca \) (see Fig. 3) is \( arbD \), a gene encoding a 290-residue protein showing high amino acid sequence identity to \( \text{Candida albicans} \) D-arabitol dehydrogenase. Centromere-proximal from \( mccB \) and separated from it by an 822-bp intergenic region is a gene predicted to encode a chitosanase, denoted \( csnA \) (Fig. 3). Similar \( csnA \) transcript levels were found on glucose, Leu, or a mixture of glucose and Leu (Fig. 3), as expected for a gene that is not involved in Leu catabolism. In contrast, Northern analysis using a probe containing the region between \( mccA \) and \( mccB \) revealed the presence of a third gene whose transcription is Leu-inducible and glucose-repressible, strongly suggesting that this region contains another gene of the Leu catabolic pathway, that was denoted \( ivdA \) (GenBank accession number AT387594). Sequence analysis of \( ivdA \) cDNA clones revealed that the gene is split by three introns and that its transcript encodes a 430-residue protein showing high amino acid sequence identity to isovaleryl-CoA dehydrogenase (IVD) enzymes (55 and 63% to human and \( \text{Pseudomonas aeruginosa} \) IVD, respectively). IVD (EC 1.3.99.10) catalyzes conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA with formation of FADH₂ (Fig. 1). The divergently transcribed \( mccB \) and \( ivdA \) genes are separated by an intergenic region of 385 bp (Fig. 3). We conclude that this region of chromosome III contains a three-gene cluster encoding two sequentially acting enzymes of the Leu degradation pathway.

**Construction and Phenotypic Characterization of \( \Delta mcca \) and \( \Delta mccB \) Alleles**—Null \( \Delta mcca \) and \( \Delta mccB \) alleles were constructed by transformation, using a gene replacement procedure. The \( mccA \) transcription start and polyadenylation sites are located at −31 (relative to the initiation codon) and +89 (relative to the stop codon), respectively. To construct a null \( \Delta mcca \) allele, a 5423-bp DNA fragment in which sequences from −14 (relative to the ATG) to +3 (relative to the stop codon) had been substituted by a 3133-bp DNA fragment containing the \( argB^+ \) gene was used to transform an \( argB2 \) arginine-requiring strain (Fig. 4A). Prototrophic transformants were purified on minus-arginine minimal medium. Of these, ∼15% were unable to grow on leucine as a sole carbon source. All 10 transformants unable to grow on leucine that were analyzed by Southern blotting showed the hybridization pattern expected for the double crossover event shown in Fig. 4A and were therefore carrying the \( \Delta mcca \) allele. Two, chosen for further analysis, were phenotypically indistinguishable in that they showed wild type growth on lactose, Ile, Phe, Pro, Thr, or Val, but their growth was severely reduced on Leu minimal medium as compared with the wild type (data not shown) (3). Northern blot analysis of mRNA isolated from cells grown under leucine-inducing conditions revealed that, in agreement with the complete deletion of the gene, the \( \Delta mcca \) mutation indeed results in undetectable levels of the \( mccA \) transcript, whereas levels of the \( ivdA \) transcript remain essentially unaffected (Fig. 5). As expected from Northern analysis, enzyme assays showed that the \( \Delta mcca \) mutation nearly abolishes MCC activity (Table I).

An apparently full-length \( mccB \) cDNA starting 39 bp upstream from the initiation codon that is polyadenylated 57 bp downstream from the stop codon was characterized. To construct a null \( \Delta mccB \) allele, a 5624-bp DNA fragment in which sequences from −21 (relative to the ATG) to +62 (relative to the stop codon) had been substituted by a 3133-bp DNA fragment containing the \( \text{Neurospora crassa} \) \( pyr-4 \) gene was used to transform a \( pyrB9 \) pyrimidine-requiring strain (Fig. 4B) (\( pyr-4 \) is the \( N. \text{crassa} \) homologue of \( A. \text{nidulans} \) \( pyrG \)). Prototrophic transformants were purified on minus-pyrimidine minimal medium and analyzed by Southern blotting and
growth tests as described above. As with ΔmccA strains, two transformants showing the expected hybridization pattern for a ΔmccB deletion event were phenotypically indistinguishable, being unable to grow on Leu as the sole carbon source, although they grew as the wild type on lactose, Ile, Phe, Thr, and Val (Fig. 4C). ΔmccB results in undetectable mccB transcript levels and, like ΔmccA, it does not affect ivdA transcription (Fig. 5) and abolishes MCC activity (Table I).

A double ΔmccA ΔmccB mutant was constructed after crossing the corresponding single mutants. As expected, whereas single mutants abolished transcription only of the corresponding deleted gene, the double ΔmccA ΔmccB mutation abolished transcription of both but had no major effect on ivdA (Fig. 5). As single ΔmccA or ΔmccB mutants (see below), ΔmccA ΔmccB strains showed residual growth on Leu, similar to that observed on agar plates lacking an added carbon source. In enzyme assays (Table I), the double mutant showed the same nearly null levels of activity than either of the single mutants. Therefore, ΔmccA, ΔmccB, and ΔmccA ΔmccB strains are phenotypically identical.

The inability of ΔmccA, ΔmccB, or ΔmccA ΔmccB strains to use Leu as the sole carbon source demonstrates the in vivo
involvement of MCC in Leu catabolism.

Fungal Model for 3-Methylcrotonyl-CoA Carboxylase Deficiency—Patients suffering from isolated MCC deficiency excrete in their urine markedly elevated amounts of 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCGly), which are diagnostic biochemical intermediates of the disease (Fig. 6, A and B) (1, 26). A. nidulans can be grown on synthetic medium containing Leu as the sole carbon source. If an amino acid degradation pathway is blocked by a loss-of-function structural gene mutation, metabolites accumulating as a result of the enzyme deficiency exit the cell and appear in the culture supernatant, which, analogously to human urine, can be analyzed by GC/MS (11, 13). In contrast to the wild type, ΔmccA ΔmccB cells accumulate 3-HIVA in a leucine-dependent manner, as expected form the specific involvement of MCC in Leu catabolism. Fig. 6, E–H, shows that the GC/MS profile of supernatants from wild type and ΔmccA ΔmccB cultures is virtually indistinguishable when cells were incubated in synthetic medium containing lactose or isoleucine as sole carbon source. In contrast, a marked accumulation of 3-HIVA was detected when the double mutant but not the wild type was cultured in medium containing leucine as the sole carbon source (Fig. 6, C and D). This marked accumulation of 3-HIVA was also detected using ΔmccA ΔmccB single mutants (data not shown). Of note, 3-MCGly was detected in neither. The specific accumulation of 3-HIVA upon culturing MCC-deficient fungal cells in Leu medium illustrates the remarkable similarity of the metabolic consequences of equivalent enzyme deficiencies in human and fungi. As in humans, 3-HIVA almost certainly results from hydration of 3-methylcrotonyl-CoA by crotonase (enoyl-CoA hydratase) followed by spontaneous decyclation (1). Although A. nidulans enzymes with enoyl-CoA hydratase activity have not yet been functionally character-
ized, the A. nidulans genome contains an almost certain candidate gene to encode such a protein. In contrast, the human plasma Gly-conjugating enzyme converting organic acids to their corresponding glycine amides would appear to be absent from A. nidulans.

2-Hydroxy-3-methylvaleric acid (2H3MV) resulting from hydration of α-keto-β-methylvaleric acid, the product of Ile transamination, accumulates in the culture supernatants of both the wild type and the MCC-deficient mutant grown in minimal medium with 10 mM Ile as the sole carbon source. This indicates that, as in humans (27), BCKA dehydrogenase activity is limiting under these conditions. BCKA dehydrogenase catalyzes the oxidative decarboxylation of all three BCAA transamination products. In agreement, a marked elevation of 2-hydroxyisocaproic acid resulting from hydration of α-ketoisocaproic acid, the Leu transamination product (Fig. 1), was seen when the Leu concentration in fungal cultures was raised to 10 mM or above (data not shown). It is worth noting that also in humans, α-ketoisocaproic acid decarboxylation is the rate-limiting step in Leu catabolism.

Whereas MCC-deficient strains grow normally on lactose, ΔmccA or ΔmccB mutations impair growth on lactose plus leucine to a significant extent, indicating that 3-HIVA and/or other metabolites accumulating in the deficient strains are toxic (Fig. 7). Since A. nidulans is an aerobe, such toxicity could reflect an impairment of mitochondrial function by these metabolites.

The Mevalonate Shunt—The biosynthetic cholesterol pathway proceeds via mevalonate formed by 3HMG-CoA reductase. Metabolic tracing studies showed that not all mevalonate is converted to sterols and strongly supported the existence of a link between the cholesterol biosynthetic pathway and Leu catabolism (see Refs. 14 and 28–30 and references therein). Edmond and Popjak (29) proposed that mevalonate is converted to acetyl-CoA via the enzymes of the Leu catabolic pathway after its conversion to 3-methylcrotonyl-CoA by the shunt pathway shown in Fig. 1. Another possible link is at the level of noncyclic isoprenoids such as geranyl-CoA, which can be catabolized by Pseudomonas citronellolis and plants by a series of reactions also leading to 3-methylcrotonyl-CoA (31, 32) (Palva, unpublished results).

Microbial or plant mutants or human patients specifically deficient in this pathway have not yet been reported, nor have animal models been devised. In view of the apparent similarity between fungal and human leucine degradation pathways, we used our fungal model deficient in MCC to obtain genetic evidence for the existence of this link, using the GC/MS procedure described above. Whereas the metabolic profile of the wild type culture supernatant was markedly similar to that of a ΔmccA ΔmccB strain when transferred to medium with 0.05% (w/v) lactose as the sole carbon source (Fig. 8, A and B), the mutant specifically accumulated 3-HIVA on transfer to acetate medium (Fig. 8, C and D) (acetate is converted to acetyl-CoA by acetyl-CoA synthetase (33)). This finding agrees with the existence of the above mentioned link, since among compounds directly derived from acetyl-CoA only isoprenoid biosynthesis intermediates can be converted to a β-methyl branched compound such as 3-methylcrotonyl-CoA. Marked accumulation of 3-HIVA in the mutant but not in the wild type supernatant was also observed upon transfer to the mevalonate precursor 3-hydroxy-3-methylglutaric acid (Fig. 8, E and F). Moreover, despite the apparently inefficient utilization of mevalonate by A. nidulans, the MCC-deficient mutant but not the wild type clearly accumulated 3-HIVA upon transfer to mevalonate (a

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TABLE I

<table>
<thead>
<tr>
<th>Protein extract</th>
<th>Specific activitya</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human fibroblasts</td>
<td>1.2 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>13.8 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td>A. nidulans wild typeb</td>
<td>25.0 ± 2.9</td>
<td>100</td>
</tr>
<tr>
<td>A. nidulans ΔmccA</td>
<td>0.15 ± 0.03</td>
<td>0.59</td>
</tr>
<tr>
<td>A. nidulans ΔmccB</td>
<td>0.12 ± 0.02</td>
<td>0.48</td>
</tr>
<tr>
<td>A. nidulans ΔmccA ΔmccB</td>
<td>0.16 ± 0.04</td>
<td>0.63</td>
</tr>
</tbody>
</table>

a Specific activities are in nmol of [14C]HCO$_3^-$/min × mg of protein.
b A. nidulans strains were grown under inducing conditions.

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*J. M. Rodríguez and M. A. Peñalva, unpublished results.*
commercial preparation that is a mixture of D- and L-isomers with mevalolactone (Fig. 8, G and H). These results strongly support the existence of a mevalonate shunt in A. nidulans.

**DISCUSSION**

Our work has pioneered the use of A. nidulans knock-out strains as fungal models for human inborn errors of metabo-
A Model for 3-Methylcrotonyl-CoA Carboxylase Deficiency

...accumulate 3-HIVA in the culture supernatant, as determined by GC/MS. 3-HIVA is one of the diagnostic compounds in the urine of MCC-deficient patients, strongly supporting the validity of the metabolic model. In the presence of lactose as the sole carbon source, the addition of Leu impairs growth of MCC-deficient strains to a significant extent, strongly indicating that one or more of the metabolites accumulating in the mutants are toxic to the cells. One possibility is that high levels of 3-HIVA accumulating in the mitochondria affect its normal functioning. A. nidulans is an aerobe, and therefore its growth rate is highly dependent on normal mitochondrial function.

Accumulation of 3-HIVA in the culture supernatant of the MCC-deficient strains is indicative of metabolite flow through the Leu degradation pathway. Growth of these strains on 0.05% lactose results in a minor yet detectable accumulation of 3-HIVA, possibly reflecting that such growth conditions result in carbon source limitation that triggers catabolism of amino acids resulting from protein turnover. The levels of 3-HIVA detected upon transfer of a MCC-deficient strain to Ile were similar to those on lactose, strongly indicating that Ile catabolism does not produce shunt metabolites that can be converted into 3-methylcrotonyl-CoA.

In humans, as much as 16.5% of mevalonate is diverted away from sterol biosynthesis (45). Both the mevalonate shunt proposed by Edmond and Popjak (29) and the geranyl-CoA catabolic pathway proposed in bacteria (31) and plants (32) involve the three final steps of Leu catabolism in the conversion of isopentenyl pyrophosphate and geranyl-CoA, respectively, into acetoacetate and acetyl-CoA. Therefore, if either or both were operative in Aspergillus, MCC-deficient strains should predictably accumulate 3-HIVA upon exposure to isoprenoid precursors. 3HMG-CoA is the immediate precursor of mevalonate, the building block of isoprenoids. The high levels of 3-HIVA found in culture supernatants of the double mec null mutant upon transfer to a medium containing 3HMG strongly indicate that a fraction of the 3HMG pool is converted into metabolites of the lower Leu catabolic pathway through isopentenyl pyrophosphate or linear isoprenoids. The finding that 3-HIVA was also detected in the mutant culture supernatant upon transfer to mevalonate strongly supports this interpretation.

A link between sterol biosynthesis and Leu catabolism is also suggested by certain biochemical findings detected in patients with Smith-Lemli-Opitz syndrome (OMIM 270400) (46) who, as a result of a deficiency of a cholesterol biosynthetic enzyme, are likely to accumulate high levels of cholesterol precursors. Patients with the lowest cholesterol levels show abnormally increased plasma levels of 3-methylglutaconic acid, as would be expected if forced flow through the mevalonate/isoprenoid shunt strains 3-MG-CoA hydratase, the enzyme downstream of MCC (47). Our work provides the first reported genetic evidence for the mevalonate/isoprenoid shunt, whose precise enzymatic basis remains to be determined.

MCC is a member of the biotin-dependent carboxylase family of enzymes. In Escherichia coli ACC, the biotin-carboxylating, biotin carrier, and carboxyltransferase domains reside in three different polypeptide chains, in contrast to fungal and mammalian ACCs, where all three activities reside in a single polypeptide. The carboxyltransferase domain is located in the β subunits, where substrate specificity resides. The high level of identity between the catalytic domains of PCCβ and MCCβ would suggest the possibility that
FIG. 8. Mevalonate shunting requires 3-methylcrotonyl-CoA carboxylase. A. nidulans wild-type or mutant ΔmccA ΔmccB strains were precultured as in Fig. 6 before analyzing organic acids extracted from culture supernatants by GC/MS. Carbon compounds were used as follows: lactose at 0.05% (w/v), acetate at 100 mM, 3-hydroxy-3-methylglutarate and mevalonate at 30 mM. Phenylacetic acid (PhAc) was added exogenously to culture supernatants and served as an internal control for the extraction procedure. Ethylvanilin (EV) was used as control for derivatization. Their peaks are not visible in the mevalonate conditions, as for G and H we injected one-fortieth of the material used for A–F, due to the fact that mevalonate appears to be inefficiently taken up by A. nidulans, which results in column saturation. 3H3MV, 3-hydroxy-3-methylvaleric acid, almost certainly proceeding from mevalonate. 2-HIVA, 2-hydroxyisovaleric acid. 3 and 8, succinic and fumaric acids, respectively. MVA, D,L-mevalonic acid; MVL, mevalolactone.
PCCα could at least partially substitute for MCCα in vivo. We show that ΔmecA, ΔmecB, and ΔmecA ΔmecB strains are phenotypically indistinguishable in their inability to grow on Leu and their nearly undetectable in vitro MCC activity. These findings rule out the above possibility and suggest that the interaction between α modules and their corresponding β subunits is specific. The structures of the biotin-carboxylating (5, 20) and biotin carrier domains (48) of ACC, the carboxyltransferase domain of yeast ACC (49), and the Propionibacterium shermanii transcarboxylase (50) have been determined. However, the structural determinants of the specific αβ interaction in PCC and MCC and the basis for substrate discrimination by their β subunits remain to be described.

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Fungal Metabolic Model for 3-Methylcrotonyl-CoA Carboxylase Deficiency
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