Defects in Mitochondrial and Peroxisomal β-Oxidation Influence Virulence in the Maize Pathogen Ustilago maydis

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An understanding of metabolic adaptation during the colonization of plants by phytopathogenic fungi is critical for developing strategies to protect crops. Lipids are abundant in plant tissues, and fungal phytopathogens in the phylum basidiomycota possess both peroxisomal and mitochondrial β-oxidation pathways to utilize this potential carbon source. Previously, we demonstrated a role for the peroxisomal β-oxidation enzyme Mfe2 in the filamentous growth, virulence, and sporulation of the maize pathogen Ustilago maydis. However, mfe2 mutants still caused disease symptoms, thus prompting a more detailed investigation of β-oxidation. We now demonstrate that a defect in the had1 gene encoding hydroxyacyl coenzyme A dehydrogenase for mitochondrial β-oxidation also influences virulence, although its paralog, had2, makes only a minor contribution. Additionally, we identified a gene encoding a polypeptide with similarity to the C terminus of Mfe2 and designated it Mfe2b; this gene makes a contribution to virulence only in the background of an mfe2Δ mutant. We also show that short-chain fatty acids induce cell death in U. maydis and that a block in β-oxidation leads to toxicity, likely because of the accumulation of toxic intermediates. Overall, this study reveals that β-oxidation has a complex influence on the formation of disease symptoms by U. maydis that includes potential metabolic contributions to proliferation in planta and an effect on virulence-related morphogenesis.
a scdA echA double mutant in Podospora anserina (4). Recent in silico surveys of the pathways encoded in more than 50 fungal genomes revealed that most fungi possess both mitochondrial and peroxisomal pathways (8, 52).

For U. maydis, we previously found that deletion of the gene encoding the peroxisomal multifunctional enzyme (Mfe2) reduced growth on long-chain fatty acids and attenuated symptom development and teliospore formation on maize (28). Defects in β-oxidation are known to influence virulence in other phytopathogenic fungi. For example, overexpression of a ketocacyl-CoA thiolase reduces the size of lesions caused by Leptosphaeria maculans on Brassica napus cotyledons and defects in β-oxidation and peroxisome biogenesis block appressorium-based invasion in Magnaporthe grisea, Colletotrichum species, and Alternaria alternata (1, 3, 12, 14, 25, 26, 46, 64). Therefore, β-oxidation represents an attractive target for the development of control strategies. In this study, we extended our analysis of β-oxidation in U. maydis through a comparative analysis of the roles of the peroxisomal and mitochondrial pathways in growth and virulence. In addition to further analysis of genes encoding the multifunctional enzyme, we targeted the had genes because defects would be expected to not only interfere with mitochondrial β-oxidation but also lead to the accumulation of toxic intermediates that might further suppress fungal growth in planta. We discovered that both pathways influence the filamentous growth response to fatty acids and both contribute to the ability of U. maydis to cause disease in maize. Overall, this work enhances our understanding of the contribution of β-oxidation to nutrient acquisition and virulence for a basidiomycete phytopathogen.

MATERIALS AND METHODS

Strains, growth conditions, and mating assay. Mutants were derived from U. maydis strains 001 (a2b2) and 002 (a1b1) and grown in potato dextrose broth (PDB), minimal medium (MM), double complete medium (DCM), potato dextrose agar (PDA), or complete medium agar (CM) (see Table S1 in the supplemental material). U. maydis cells were spotted on double complete medium with 1% activated charcoal (DCM-C) to assay mating (10, 20, 29).

Identification of β-oxidation genes and construction of deletion strains. The β-oxidation genes in U. maydis were identified from the genome sequences at the Broad Institute (http://www.broadinstitute.org/scientific-community/data). The mfe2 gene of U. maydis (UM00150; alternative name, Um10038) was previously identified by Klose and Kronstad (28), and mfe2b (UM01747) was identified by BLASTp analysis with the A chain of the human Mfe2 protein (NCBI 1S9C_A, GI 61679854). The mitochondrial β-oxidation genes were identified with the human Had protein (3-hydroxyacyl-CoA dehydrogenase; NCBI CA465528.1, GI 1483511). U. maydis has two homologues: had1 (UM01099) and had2 (UM02105).

An overlap PCR strategy was used to delete the mfe and had genes and to generate the mutants listed in Table S1 in the supplemental material (9). Briefly, ~1 kb of 5’ upstream sequence (left arm) and ~1 kb of 3’ downstream sequence (right arm) were amplified along with a selectable marker from a plasmid containing the resistance cassette. The three parts were combined via overlap PCR to replace the original open reading frame of the gene with the resistance marker. The deletion construct was reamplified with nested primers and used for biolistic transformation as described by Toffaletti et al. (56). U. maydis transformants were grown overnight on DCM with 1 M sorbitol and then transferred to DCM agar containing 150 μg/ml−1 nourseothricin or 250 μg/ml−1 hygromycin B for selection. Gene deletion was confirmed by colony PCR and genomic hybridization. Gel electrophoresis, restriction enzyme digestion, and Southern blot hybridization were performed using standard procedures (50). To delete the U. maydis mfe2b gene, flanking regions were amplified with primers UmMfe2b1 and UmMfe2b2 and primers UmMfe2b5 and UmMfe2b6, resulting in the left and right arms of the deletion construct, respectively. The sequences of all primers are given in Table S2 in the supplemental material. The nourseothricin resistance marker was amplified with primers UmMfe2b3 and UmMfe2b4 from plasmid pMF1-n (see Table S3 in the supplemental material). The final construct was amplified with nested primers UmMfe2b7 and UmMfe2b8, resulting in a deletion construct of 3.0 kb.

To delete the U. maydis had1 gene, the left arm and right arm were amplified using primers Had1P1 and Had1P2 and primers Had1P5 and Had1P6, respectively. The 1.8-kb nourseothricin resistance marker was amplified from plasmid pSAT12 using primers Had1P3 and Had1P4. The three fragments were combined by an overlapping PCR using primers Had1P1 and Had1P6, resulting in a 3.4-kb deletion construct. The deletion strain 001 had1Δ was generated by biolistic transformation. The deletion mutant for had1 in the a1b1 mating-type background was generated by a sexual cross of strain 001 had1Δ and the wild-type strain 002 (a1b1) (19). Teliospores harvested from maize seedlings inoculated with the cross of the 002 × 001 had1Δ strains were germinated on PDA at 30°C for 24 h. The haploid progeny carrying the had1Δ selection were selected on CM agar containing nourseothricin, and the mating types of the progeny were determined in tests with the 001 and 002 strains.

The had1Δ mfe2a double mutants were generated by a sexual cross of mfe2Δ mutant 6 (002) and had1Δ mutant 34-1 (001). Spores recovered from infected plants were initially germinated on CM with 150 μg ml−1 nourseothricin, with subsequent transfer to CM with 250 μg ml−1 hygromycin B to select the double mutants. The presence of the mutations was confirmed by PCR. To determine the mating type of the strains, a PCR was performed with primers Ummfa2_f, Ummfa2_r, Ummfa1_f, and Ummfa1_r for the a locus, and PCR-restriction fragment length polymorphism analysis was employed for the b locus with primers Umb_f and Umb_r, as described by Zambino et al. (66). Three double mutants were used for subsequent analysis.

To delete the U. maydis had2 gene, the left and right arms of the deletion construct were amplified with primers UmHad2_1 and UmHad2-2hyg and primers UmHad2_5hyg and UmHad2_6, respectively. The hygromycin B resistance marker was amplified with primers UmHad2_3hyg and UmHad2_4hyg from plasmid pC19RHL. The final construct was amplified with nested primers UmHad2_7 and UmHad2_8, resulting in a deletion construct of 4.1 kb.

RNA extraction. Fungal cells were grown overnight in 5 ml of PDB and washed once with sterile water, and 1 × 107 cells were transferred to 5 ml of MM supplemented with either glucose, caproic, myristic, oleic, or linoleic acid (all added to 1%, except for caproic acid, which was added to 0.1%). The cells were then grown at 30°C at 250 rpm for 6 h, 5 × 107 cells were harvested and washed, and RNA was extracted with a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). For the analysis of glucose repression, myristic acid and glucose were added at a final concentration of 1% each.

To examine the expression of the β-oxidation genes in planta, the strain 001 and 002 cells for plant inoculation were grown in PDB, washed once in sterile water, and mixed in a 1:1 ratio with a final concentration of 1 × 106 ml−1 for the 24-h infection stage and 1 × 108 ml−1 for the 7-day and 21-day infection stages. Infected plant tissue was harvested at 0 h (control), 24 h, 7 days, and 21 days. RNA was isolated from these stages and from a mating culture with the Qiagen RNeasy kit after the tissue in a mortar and pestle was cooled with liquid nitrogen.

PCR analysis of RNA expression. For quantitative PCR, DNase I-treated total RNA was used to generate oligo(dT)-primed cDNA (Verso cDNA kit; Thermo Fisher Scientific, Waltham, MA). cDNA corresponding to 5 ng of total RNA and 2.5 pmol of each primer were used in each reaction. The real-time PCR was performed with Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA) in a 20-μl reaction mixture on an Applied Biosystems platform. Primers are listed in Table S3 in
the supplemental material. The size of the PCR product was confirmed for each reaction by gel electrophoresis. Gene expression was calculated relative to the constitutive expression of the genes for actin and glyceraldehyde phosphate dehydrogenase from U. maydis.

**Growth assays on fatty acids.** To characterize the morphological response and to quantitate growth on fatty acids, cells were washed once with sterile water and 1 × 10⁶ cells were added to 5 ml of liquid MM supplemented with glucose, butyric acid (4:0), caproic acid (6:0), lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), oleic acid (18:1(n-9)), or linoleic acid (18:2(n-6)) (Sigma-Aldrich, St. Louis, MO) as the sole carbon source (at a concentration of 1% for all supplements except for butyric and caproic acid [0.01%] and lauric acid [0.1%]). To examine the accumulation of toxic intermediates, glucose was replaced by 1% arabinose, a carbon source without catabolite repression activity (28). The cells were grown at 30°C for 5 days with shaking at 250 rpm. The extent of growth was determined by cell counts with a hemacytometer. Cell morphology was visualized by staining with 1 µl fluorescent brightener 28 calcicolour white (20 µg ml⁻¹), which was added directly to 5 ml of culture on a microscope slide. Cells were observed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany) with UV fluorescence for cells stained with calcicolour white. Images were captured with a digital video camera (DVC) and processed with Adobe Photoshop 7.

**Influence of β-oxidation on stress susceptibility.** To examine stress and drug responses, exponentially growing cultures of U. maydis were washed with MM, resuspended in the same medium, and adjusted to 2 × 10⁶ cells ml⁻¹. After 10-fold serial dilutions, 5 µl of each dilution was spotted onto PDA or MM plates. The following inhibitors and agents were added to MM at the indicated concentrations to provoke stress: 1.2 M NaCl, 0.5 mg ml⁻¹ caffeine, 100 µM tert-butyl hydroperoxide, 25 µg ml⁻¹ brefeldin A (BFA), 250 µM monensin, 5 µg ml⁻¹ or 10 µg ml⁻¹ fluconazole, and 10 mM LiCl. The plates were incubated for 2 to 4 days at 30°C or 37°C and photographed. Ergosterol sensitivity was tested on PDA plates with 100 µg ml⁻¹ ergosterol. Sensitivity to ethanol (EtOH) was tested in a 96-well plate with 100 µl PBD with increasing concentrations of ethanol ranging from 0.1% to 10%. Five thousand cells were inoculated per well, the plates were incubated for 48 h at 30°C in a humid chamber, and the MIC was determined.

**Cell death assay.** Cells were grown in PDB, washed in MM, and incubated in 5 ml MM with different stressors for 2.5 h at 30°C and 250 rpm. MM with glucose was used as a control and to test BFA (25 µg ml⁻¹), while for fatty acids, the glucose was replaced by 1% caproic acid. The cells were centrifuged, washed once in KC (0.6 M KCl, 50 mM CaCl₂), and protoplasted for 20 min in 1 ml KC with 100 mg lytic enzymes at room temperature (Sigma-Aldrich, St. Louis, MO). The protoplasts were centrifuged at 1,600 × g for 5 min at 4°C and washed three times with ice-cold KC. The cellular components were fixed for 1 h in 1.85% formaldehyde in KC. After washing in KC, the cells were permeabilized with ice-cold KC containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The cells were washed with KC and phosphate-buffered saline (PBS) and incubated for 1 h at 37°C in the dark with 50 µl of the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) mixture (in situ cell death detection kit, fluorescein; Roche Applied Science, Laval, Quebec, Canada). The cells were washed twice in PBS prior to microscopic observation. The cells were mounted with Prolong antifade gold (Invitrogen, Burlington, Ontario, Canada) with DAPI (4’,6-diamidino-2-phenylindole) and immediately analyzed with a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany). With differential interference contrast, DAPI, and a fluorescein isothiocyanate filter for fluorescein. Images were captured with a DVC and processed with Adobe Photoshop (version 7) software. Controls included untreated cells incubated in the TUNEL mixture without enzyme, untreated cells incubated in the mixture with enzyme, and cells treated with DNase I for 15 min before incubation in the TUNEL mixture with enzyme.

**Virulence assays.** The virulence of U. maydis strains was determined by inoculation of 7-day-old maize seedlings with 100 µl of a mating mixture of the 001 and 002 wild-type strains or mutants at a cell concentration of 1 × 10⁶ ml⁻¹. Disease symptoms were evaluated as described previously (28). A total of 60 to 70 plants were infected in three independent experiments with each combination of strains.

**Statistical analysis.** The data are representative of at least three independent experiments. Values are given as the mean of triplicates ± standard deviation. The t test was used for statistical analysis.

**RESULTS**

**Genes for both peroxisomal and mitochondrial β-oxidation are present in U. maydis.** Our previous characterization of an mfe2Δ mutant lacking the multifunctional enzyme in U. maydis indicated a role for peroxisomal β-oxidation in virulence on maize (28). The mfe2Δ gene is highly conserved in fungi and animals, although the polypeptide does not have clear peroxisomal targeting sequences (e.g., PTS1 or PTS2; http://www.peroxisomedb.org/Target_signal.php). The closest fungal homologues are found in Malasseziaglobosa, Puccinia graminis, and Laccaria bicolor (see Table S4 in the supplemental material). Given that an mfe2Δ mutant still caused attenuated disease in maize, we hypothesized that additional β-oxidation functions must be present in U. maydis. A reexamination of the U. maydis genome identified a candidate for a second mfe2 gene, designated mfe2b, of lower sequence similarity (1.7e⁻⁵⁴ compared to the sequence of Mfe2 from U. maydis). Mfe2b has a predicted peroxisomal PTS1 sequence (ALK; http://www.peroxisomedb.org/Target_signal.php). The closest homologues to Mfe2b in the basidiomycetes are found in Schizopyllum commune, Coprinopsis cinerea, and L. bicolor; homologues were also found in ascomycete fungi and in animals (see Table S1 and Fig. S1 in the supplemental material).

Inspection of the U. maydis genome also revealed candidate genes for at least two enzymes for each of the four steps involved in mitochondrial β-oxidation. The hydroxyacyl-CoA dehydrogenase enzymes Had1 and Had2 that carry out the third step were selected to examine their role in virulence because a defect at this step would potentially block β-oxidation and cause the accumulation of toxic intermediates. Had1 has a signal peptide for mitochondrial targeting (clevage at amino acid 39, P = 0.98; http://ihg.gsdf.org/ihg/mitoprot.html). The phylogeny of had1 is interesting because no close homologues were found in ascomycete fungi or in bacterial species. The closest homologues are found in M. globosa, Cryptococcus neoformans, and Haliotis discus (see Table S1 and Fig. S2 in the supplemental material). A third basidiomycete homologue can be found in the rust fungus P. graminis. Had2 of U. maydis did not have an identifiable mitochondrial signal peptide, although one is present in the orthologue in C. neoformans (http://ihg.gsdf.org/ihg/mitoprot.html). Had2 shows the highest homology to C. cinerea, S. commune, and L. bicolor, and similar enzymes were evident in bacteria and ascomycetes.

**Fatty acid induction and glucose repression of β-oxidation genes.** We initially examined transcript levels for the peroxisomal (mfe) and mitochondrial (had) genes and found that they were upregulated in the presence of exogenous fatty acids, relative to growth in glucose (Fig. 1A and B). Induction occurred regardless of the chain length or saturation state of the fatty acids. In addition, the transcripts of the peroxisomal genes were generally more highly induced than those of the mitochondrial genes, and this was particularly marked for mfe2b (Fig. 1A). The cyclooxygenase-encoding gene ssp1 from U. maydis was included as a control because of its connection to lipid metabolism and its elevated ex-
Expression of β-oxidation genes during infection. The waxy plant surface is a nutrient-poor environment for phytopathogens, and knowledge about specific nutritional requirements during growth in planta is limited (53). We therefore examined the expression of β-oxidation genes during the infection of maize seedlings by U. maydis (Fig. 1C). At early stages of maize infection (24 h), the genes showed elevated expression ranging from 5.6-fold for had1 to 19.3-fold for had2, relative to the expression of the genes in inoculated cells (grown in PDB). Lower expression was detected at 7 days after inoculation (e.g., 1.8-fold for had2 and 3.4-fold for mfe2b) and at day 21 during sporulation (e.g., 2.7-fold for mfe2b and 4.8-fold for had2). The transcript for sspl showed co-regulation with the β-oxidation genes, as expected from its documented expression during infection (22). Overall, the transcripts of the β-oxidation genes in U. maydis were expressed at elevated levels during infection, relative to the levels under specific in vitro growth conditions.

Deletion of β-oxidation genes influences the growth of U. maydis on fatty acids. We next constructed mutants lacking the peroxisomal (mfe2b) and mitochondrial (had1 and had2) β-oxidation genes (see Fig. S3 in the supplemental material) and compared these mutants with the previously constructed mfe2Δ mutant for growth on fatty acids, virulence-related phenotypes, and virulence. As shown in Fig. 2, the wild-type strain of U. maydis utilized a broad range of fatty acids for growth, including short-chain (C4 to C6), medium-chain (C12), long-chain (C14 to C18), and polyunsaturated, long-chain (e.g., C18:2) fatty acids. The chemical structures of the fatty acids are shown in Fig. S4 in the supplemental material. The cells grew to the highest density on myristic and oleic acids. The short-chain fatty acids butyric acid (C4) and caproic acid (C6) appeared to be toxic to U. maydis at 1% (see below), although a lower concentration (0.01%) supported weak growth. The medium-chain fatty acid lauric acid (C12) also showed toxicity at a concentration of 1%, but growth was supported at a lower concentration (0.1%). Our previous analysis in U. maydis also demonstrated poor growth of wild-type and mfe2Δ strains on these fatty acids (28). For the mutants, we generally observed that deletion of both genes in the peroxisomal (i.e., mfe2Δ and mfe2bΔ) or the mitochondrial (had1Δ and had2Δ) pathways (to establish a complete block) had a greater effect on the ability to use fatty acids than deletion of a single gene (e.g., mfe2Δ or had1Δ) or one gene in each pathway (mfe2Δ and had1Δ). This suggests the presence of functional redundancy between the proteins Mfe2 and Mfe2b or Had1 and Had2. In addition, the peroxisomal enzyme Mfe2 and the mitochondrial enzyme Had1 made larger contributions to growth on fatty acids relative to those of Mfe2b and Had2, respectively (Fig. 2).

Analysis of the growth of the mutant blocked in the mitochondrial pathway (had1Δ had2Δ) revealed defects on short-chain (C4 to C6) and medium-chain (e.g., lauric acid [C12]) fatty acids and a lesser contribution on fatty acids with greater chain length; these genes also made only a very minor contribution to growth on the long-chain, unsaturated fatty acid oleic acid (C18:1). However, the mitochondrial pathway was also involved in utilization of the long-chain, polyunsaturated fatty acid linoleic acid (C18:2). For mutants with defects in single genes, had1Δ was partially involved in the utilization of capric acid, lauric acid, myristic acid, and linoleic acid, while had2Δ played only a minor role in the utilization of lauric acid, palmitic acid, and linoleic acid. In contrast, the peroxisomal pathway (mfe2Δ mfe2bΔ) played no role in the utili-

Expression of peroxisomal and mitochondrial β-oxidation genes. Cells were grown in MM with caproic acid (cap; 0.1%), myristic acid (myr; 1%), oleic acid (olei; 1%), linoleic acid (lino; 1%), or glucose (glu; 1%) for 6 h. The genes in all graphs are as follows: mfe2, mfe2b, had1, had2, and sspl. (A) Quantitative PCR analysis of transcript levels for the β-oxidation genes. Cells were pregrown for 48 h in MM with 1% myristic acid as the carbon source, washed in water, and transferred to MM with either 1% myristic acid alone, myristic acid and glucose, or glucose alone for 6 h. The quantitative PCR analysis of glucose repression of transcript levels for the β-oxidation genes is shown. The horizontal line in each graph shows the threshold for a 2-fold change.

**FIG 1** Expression of peroxisomal and mitochondrial β-oxidation genes. (A) Quantitative PCR analysis of transcript levels upon growth on fatty acids. Cells were grown in MM with caproic acid (cap; 0.1%), myristic acid (myr; 1%), oleic acid (olei; 1%), linoleic acid (lino; 1%), or glucose (glu; 1%) for 6 h. The genes in all graphs are as follows: mfe2, mfe2b, had1, had2, and sspl. (B) Quantitative PCR analysis of glucose repression of transcript levels for the β-oxidation genes. Cells were pregrown for 48 h in MM with 1% myristic acid as the carbon source, washed in water, and transferred to MM with either 1% myristic acid alone, myristic acid and glucose, or glucose alone for 6 h. (C) Quantitative PCR analysis of transcript levels for β-oxidation genes on maize plants infected with U. maydis. RNA was isolated at 0 h, 24 h, 7 days, and 21 days postinfection. The mean of three independent experiments with standard deviation is shown. The horizontal line in each graph shows the threshold for a 2-fold change.

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zation of short-chain fatty acids. However, growth was severely compromised on fatty acids with longer chain lengths (C12 to C18). For the mutants with single mutations, \( mfe2 \)/H9004 was mainly required for utilization of lauric acid, oleic acid, and linoleic acid and partially for myristic acid and palmitic acid, while \( mfe2b \)/H9004 alone made no notable contribution. However, in the background of the \( mfe2 \)/H9004 defect, \( mfe2b \)/H9004 makes a contribution to the utilization of myristic acid and palmitic acid. This is interesting because Mfe2b lacks the hydroxyacyl dehydrogenase domains found in Mfe2, and whether another peroxisomal enzyme provides this activity for myristic and palmitic acids in the absence of Mfe2 therefore remains to be discovered (see Fig. S1 in the supplemental material).

Short- and medium-chain fatty acids are toxic, induce cell death, and lead to accumulation of toxic intermediates during \( \beta \)-oxidation. The inability of cells to catabolize fatty acids can lead to the accumulation of toxic fatty acids or their metabolites (13, 23, 31, 35, 36, 40). In this regard, short- and medium-chain fatty acids at a concentration of 1% were unable to support the growth of \( U. \) maydis, although lower concentrations supported growth (as mentioned earlier). Furthermore, we were unable to isolate RNA from cells treated for 6 h with high concentrations of these fatty acids although we were able to obtain RNA from cells treated with lower concentrations. These observations support the possibility of toxicity of short- and medium-chain fatty acids for \( U. \) maydis.

To investigate this possibility, a TUNEL assay was performed on wild-type cells incubated in the presence or absence of caproic acid. We found that 48.4% of the fatty acid-treated cells but only 1.9% of the control cells showed DNA laddering, as indicated by the TUNEL staining (Fig. 3A). Additionally, treatment with caproic acid drastically reduced the generation of protoplasts by lytic enzymes (Fig. 3A and data not shown). Taken together, these observations suggest that caproic acid triggers cell death as well as cell wall changes that interfere with RNA isolation and the enzymatic activity of cell wall lytic enzymes.
To test the possibility that a block in β-oxidation caused fatty acid toxicity or the accumulation of toxic intermediates, wild-type, mfe2Δ mfe2bΔ, and had1Δ had2Δ cells were incubated in the non-catabolite-repressing sugar arabinose with or without fatty acids (Fig. 3B). The prediction was that toxic intermediates may accumulate in the presence of fatty acids and reduce growth in medium with arabinose compared with the level of growth in medium with arabinose alone. Interestingly, the addition of lauric acid to medium with arabinose resulted in the reduced growth of the wild-type strain compared with that in medium with arabinose alone, although the level of growth was similar to that seen in medium containing only lauric acid (Fig. 3B). This suggested that lauric acid has toxicity in the presence of arabinose even for the wild type (Fig. 3B). Both lauric acid and oleic acid greatly reduced the growth of the mfe2Δ mfe2bΔ double mutant in arabinose, suggesting the accumulation of toxic intermediates. We also analyzed the contribution of the single mutations mfe2Δ and mfe2bΔ to this toxic effect and found that only the mfe2Δ mutant showed reduced growth on arabinose with lauric acid. The mfe2bΔ mutant behaved like the wild-type strain (data not shown). Accumulation of toxic intermediates was also observed for the had1Δ had2Δ mutant on lauric acid but not oleic acid. Overall, these results support the possibility that toxic intermediates accumulate when β-oxidation is blocked.

Defects in mitochondrial and peroxisomal β-oxidation block acetate utilization. A defect in the second enzyme (Fox2) for peroxisomal β-oxidation in Candida albicans is known to interfere with glyoxylate pathway function and growth on non-fermentable carbon sources (42, 43). We therefore examined the growth of the U. maydis β-oxidation mutants on MM with acetate (C2), lactic acid (C3), glycerol (C3), arabinose (C5), or citrate (C6). Growth differences were mainly observed on acetate, as shown in Fig. 4. In particular, the mfe2Δ mfe2bΔ mutant showed weak growth on glycerol, on MM with glucose, and on the more complex medium PDA, but this mutant showed practically no growth on acetate. It is known that biotin synthesis in fungi involves 8-amino-7-oxononanoate (AON) synthase and that this enzyme is located in the peroxisome. Furthermore, precursors of biotin synthesis such as pimelic acid depend on β-oxidation activity (38). Thus, we tested if a block in the peroxisomal β-oxidation pathway led to a biotin autotrophy and found that addition of 1 μM biotin to the minimal medium was not able to rescue the growth phenotype of the mfe2Δ mfe2bΔ mutant (Fig. 4). Therefore, additional work is needed to explore the relationship between biotin synthesis and β-oxidation in basidiomycete fungi. Surprisingly, the had1Δ mutant was also defectve for growth on acetate, and these results therefore suggest that the glyoxylate pathway is dependent on the functions of both β-oxidation pathways in U. maydis (Fig. 4).

FIG 4 β-Oxidation is required for growth on acetate. The growth of the single and double β-oxidation mutants and the wild-type strain on MM or PDA medium with glucose was compared with that on acetate or glycerol (1%). Minimal medium supplemented with 1 μM biotin did not rescue the growth phenotype of the mfe2Δ mfe2bΔ mutant. We also examined the rescue activity of biotin for the mfe2Δ mutant in C. neoformans and saw a minor rescue activity (28a). We cannot exclude the possibility of a very weak activity of biotin for U. maydis, which was undetectable in this assay. The plates were incubated for 3 days at 30°C.

A defect in mitochondrial β-oxidation results in resistance to the secretion inhibitor brefeldin A. Defects in peroxisomal function and β-oxidation could alter the composition of plasma membrane phospholipids due to changes in the availability of specific fatty acids or through altered catabolism of membrane-derived fatty acids. For example, Lockshon et al. (32) found that a peroxisomal defect in S. cerevisiae caused oleate sensitivity and proposed that this phenotype arose from an altered fatty acid composition of membrane phospholipids. In this context, membrane changes could impair the response of β-oxidation mutants to environmental stresses and to agents that perturb membrane and cell wall integrity. We therefore tested the mutants for growth at 37°C and in the presence of NaCl, LiCl, fluconazole, tert-butyl hydroperoxide, monensin, caffeine, BFA, EtOH, and ergosterol. As shown in Fig. 5, the only phenotype observed was reduced sensitivity to the secretion inhibitor BFA for the had1Δ mutant (Fig. 5A). That is, no differences were found for any mutant compared to the wild-type parental strain for high temperature or on media containing the other agents (data not shown).

BFA is an inhibitor of trafficking between the endoplasmic reticulum (ER) and the Golgi apparatus, and the insensitivity of the had1Δ mutant may be due to reduced uptake, perhaps because of altered plasma membrane composition, altered trafficking of target proteins, or changes in internal membrane structures in the cell. However, it is also possible that BFA triggers cell death in cells via a process that is dependent on mitochondrial β-oxidation because connections between BFA, apoptosis, and mitochondrial function are known in other organisms (17, 51, 65). To test this idea, we used a TUNEL assay to examine DNA fragmentation as a hallmark of cell death in the wild-type strain and the had1Δ mutant upon treatment with BFA. We did not observe a difference between untreated and BFA-treated wild-type cells (1.91% versus 3.83%) or the had1Δ mutant (2.64% versus 2.51%) (Fig. 5B). These analyses were performed in parallel with the TUNEL assays for caproic acid-treated cells, in which we observed positive staining for DNA fragmentation (Fig. 3). Therefore, the toxicity of BFA for the wild-type strain does not appear to result from induction of cell death, and further study is needed to identify the mechanism of reduced sensitivity for the had1Δ mutant.

With regard to other possible phenotypes, Maggio-Hall and Keller (35) reported that a defect in mitochondrial β-oxidation (i.e., loss of echA) blocks the utilization of valine and isoleucine as...
carbon sources by *A. nidulans*. However, these amino acids and leucine do not serve as sole carbon sources to support the growth of wild-type strains of *U. maydis*, and we therefore did not test the β-oxidation mutants on these amino acids.

**β-Oxidation influences filamentous growth in *U. maydis*.** We previously showed that *U. maydis* is able to switch from yeast-like growth to filamentous growth in response to lipids and fatty acids and that the *mfe2* gene is required for this response (27, 28). We therefore extended the analysis of the filamentation response to include the *mfe2b* mutant, except the *mfe2b* mutant showed the same growth pattern as the wild-type strain (Fig. 6)(28). In general, defects in both the peroxisomal and mitochondrial β-oxidation pathways influenced both the growth on particular fatty acids and the ability of the fatty acids to trigger the switch from yeast-like to filamentous growth. Also, the transition to filamentous growth was independent of the chain length and the saturation state of the fatty acids (Fig. 6), and acetate was not able to induce the transition (data not shown). As seen previously for the wild-type strains and the *mfe2* mutant, none of the strains were able to switch to filamentous growth on caproic acid (Fig. 6) (28). The *mfe2* mutant grew poorly on lauric, oleic, and linoleic acids and was unable to switch to filamentous growth in response to these fatty acids. However, the mutant was able to grow and switch on myristic and palmitic acids (Fig. 6). In contrast, the *mfe2* mutation did not have a significant influence on the growth on any fatty acid as the sole carbon source (Fig. 2), and the mutant behaved like the wild-type strain in the switch to filamentous growth (data not shown). The *had2* mutant did not grow on lauric or linoleic acid and also failed to switch to filamentous growth when grown on these fatty acids (Fig. 6). The *had2* mutant showed the same growth pattern as the *had1* mutant, except that it was able to grow and switch to a filamentous morphology on lauric acid. Double mutants with the *mfe2* and *had1* mutations showed the combined phenotypes of the single mutants (data not shown). While the loss of the peroxisomal β-oxidation pathway in the *mfe2* and *mfe2b* double mutant abolished the growth on all fatty acids with a length greater than C8, it also abolished the switch to filamentous growth for these fatty acids (Fig. 6). The block in the mitochondrial β-oxidation pathway due to loss of both *had1* and *had2* also reduced growth and filamentation on butyric acid (Fig. 6). Furthermore, no growth was observed on lauric, myristic, and linoleic acids, and no switch to filamentous growth was observed for these fatty acids (Fig. 6). The switch to filamentous growth generally did not involve all cells in a culture, and the proportion of filamentous cells depended on the fatty acid, with the highest level seen for oleic acid (>50%).

Filamentous growth as a result of mating is required for the pathogenicity of *U. maydis*. Therefore, all of the β-oxidation mutants were tested for their ability to mate with a wild-type strain of the opposite mating type or with the corresponding strain carrying the same mutation (Fig. 7 and data not shown). All mutants except the *mfe2* and *mfe2b* double mutant showed normal mating reactions in combination with the wild-type strain of the opposite mating type, as indicated by the formation of white mycelial growth on mixed colonies. The *mfe2* and *mfe2b* double mutant showed reduced mating with a compatible wild-type strain and weak mating when mixed with an *mfe2* and *mfe2b* mutant of the opposite mating type. The *had1* and *had2* double mutant also had slightly reduced mating when mixed with a double mutant of the opposite mating type (Fig. 7).

**Both mitochondrial β-oxidation and peroxisomal β-oxidation influence virulence in *U. maydis*.** The *U. maydis* β-oxidation genes were expressed during infection, suggesting that fatty acid catabolism may be important for growth in planta (Fig. 1C). In addition, previous work with *mfe2* in *U. maydis* indicated a role in virulence (28). To examine the contribution of β-oxidation in more detail, we inoculated maize seedlings with a mixture of the compatible wild-type strains and/or the different β-oxidation mutants and found that inoculation with wild type resulted in a
typical range of disease symptoms from completely health to death (disease index, 3.5 out of 5.0; Fig. 8). The majority of the plants showed severe symptoms that included large tumors on plant stems and death. No differences in virulence were observed for infections with the combination of mutant mfe2b/H9004 with mutant mfe2b/H9004, mutant had2/H9004 with mutant had2/H9004, or mutant had2/H9004 with the wild-type strain compared with the wild-type infections (Fig. 8). In contrast, infections with combinations of strains lacking had1/H9004 (had1/H9004 mutant × had1/H9004 mutant), mfe2Δ (mfe2Δ mutant × mfe2Δ mutant), or both had1Δ and mfe2Δ (had1Δ mfe2Δ mutant × had1Δ mfe2Δ mutant) resulted in less severe symptoms and a lower disease index (disease index, 2.3 to 2.5) (Fig. 8). This shift in the disease index resulted from a higher number of healthy plants and a lower number of dead plants, although the range of disease symptoms was not drastically changed between the plants inoculated with the mutants and those inoculated with the wild-
enced growth on linoleic acid (C18:2). This result is reminiscent of defects in both mitochondrial and peroxisomal contribution of the mitochondrial pathway. This makes a contribution to virulence, and they reveal an additional way in the processing of long-chain fatty acids. These patterns are similar to the organelle contributions observed in mammalian cells and A. nidulans (23, 35–37, 47, 61, 62).

We did observe that defects in both mitochondrial and peroxisomal β-oxidation influenced growth on linoleic acid (C18:2). This result is reminiscent of the partnership of the two pathways to accomplish full growth on long-chain and very-long-chain fatty acids in A. nidulans (35).

The expression patterns for the β-oxidation genes and their involvement in growth on fatty acids were similar to observations in other fungi. Other phenotypes such as the weak growth of the had1Δ and mfe2Δ mutants on acetate resembled the situation in some fungi but not others. For example, in A. nidulans, a foxA mutant with a defect in peroxisomal β-oxidation and an echA mutant with a defect in mitochondrial β-oxidation were able to grow on acetate (35). However, a C. albicans mutant lacking peroxisomal β-oxidation (fox2Δ/fox2Δ) is defective for growth on acetate (42, 43). This study also suggested that an accumulation of acyl-CoAs due to a loss of Fox2 may interfere with peroxisomal transport proteins necessary to transport glyoxylate cycle substrates and/or acetyl-CoA. A similar inhibitory influence of acyl-CoAs on mitochondrial carrier proteins has been characterized, and this process may also account for the inability of the U. maydis had1Δ mutant to grow on acetate (41). This observation may also reflect interactions between β-oxidation functions and other activities in the mitochondria. For example, a connection between hydroxyacetyl-CoA dehydrogenase activity and the respiratory electron transport chain has been described in rat liver mitochondria, and enzymes of β-oxidation are physically associated with oxidative phosphorylation complexes (55, 63). A defect in a key mitochondrial process, such as oxidative phosphorylation, would explain the poor growth of the had1Δ mutants on acetate. The had1Δ mutant also had the interesting phenotype of reduced sensitivity to BFA, an inhibitor of protein secretion between the ER and the Golgi apparatus as well as retrograde transport from the Golgi apparatus to the ER. BFA is known to induce apoptosis in a mitochondrial-dependent manner, but we were unable to detect cell death in U. maydis in response to BFA, although we did detect it in response to caprylic acid (17, 51, 65). It is possible that deletion of had1 resulted in reduced uptake of BFA, and additional analysis is needed to investigate this idea. We also observed poor growth of the mfe2Δ mfe2bΔ double mutant on acetate and on solid medium with glycerol or glucose as the carbon source, although there was no growth defect in liquid medium. The severity of peroxisomal dysfunction or the extent of accumulation of in-

FIG 8 β-Oxidation is required for full virulence of U. maydis. The virulence of U. maydis β-oxidation mutants was tested in a maize seedling infection assay. Disease development was scored at 14 days after inoculation, according to the following disease classes: no symptoms (0 = white), anthocyanin production (1 = yellow), small leaf tumors (2 = light orange), small stem tumors (3 = orange), large stem tumors (4 = orange-brown), and plant death (5 = brown). The average disease index from three independent experiments was calculated, and 60 to 70 plants were inoculated for each strain combination. The disease index (DI) and standard deviation are indicated above each bar.
hibitory metabolic intermediates may be dependent on culture conditions, such as the extent of oxygen availability.

A mutant lacking the had1 and had2 genes showed poor growth on lauric acid with or without the addition of arabinose as a nonrepressing additional carbon source. As observed in A. nidulans (23, 35, 36), this is likely due to the accumulation of toxic intermediates resulting from the block at the third step in mitochondrial β-oxidation. We did observe, however, that a wild-type strain also shows poor growth on lauric acid plus arabinose and that certain fatty acids are toxic for U. maydis. Interestingly, we also found that a mutant defective in the second and third steps (mfe2Δ mfe2bΔ) of the peroxisomal β-oxidation pathway had poor growth in the presence of oleic acid plus arabinose, indicating a potential accumulation of toxic intermediates. Importantly, these results support the possibility that targeting β-oxidation may have utility for blocking fungal disease in plants, particularly given that loss of peroxisomal or mitochondrial β-oxidation pathway activity results in three phenotypes that could potentially limit growth: a defect in fatty acid utilization as a carbon source, loss of acetate metabolism, and accumulation of toxic intermediates. It is also possible that the enzymes for β-oxidation in plants, which occurs primarily in the peroxisome, may be sufficiently distinct to allow specific targeting of the pathogen activities (15).

Our virulence assays support the idea that blocking β-oxidation could suppress U. maydis disease in planta. Specifically, deletion of the had1 or mfe2 gene or deletion of both genes together resulted in a reduction in symptoms and plant death (Fig. 8) (28). A further reduction in virulence was seen in double mutants lacking both had genes (had1Δ had2Δ) or both mfe2 genes (mfe2Δ mfe2bΔ). The latter double mutant showed the greatest defect, with very few tumors and dead plants. Although loss of mfe2 did not result in reduced mating, our previous study indicated that plant colonization was reduced (28). Because the mfe2Δ mfe2bΔ mutant showed reduced growth on minimal medium, which might contribute to virulence attenuation, and also reduced mating in vitro, we assume that mating and proliferation of the filamentous cell type in planta are strongly compromised in this mutant. In contrast, the had mutants still displayed a robust mating reaction in culture, suggesting that virulence attenuation resulted from defects after formation of the infectious cell type, comparable to the situation with the mfe2Δ mutant (28).

The ability of U. maydis to switch from yeast-like to filamentous growth is regulated by mating, pH, low nitrogen, phosphate, and fatty acids (5, 27, 28, 34, 49). We previously showed that loss of mfe2 reduced growth and filamentation on fatty acids (28). Although similar trends were seen with the same mfe2Δ mutant in this study, we noted variability in the amount of growth of the wild-type strains on myristic and palmitic acids, and this may reflect the poor solubility of the fatty acids in liquid culture media. However, the properties of the different fatty acids (ranging from liquid and water-soluble [e.g., butyric acid] to crystals and insoluble [e.g., palmitic acid]) used in this study and concerns about interference prompted us to avoid the use of solvents. Thus, we performed the fatty acid growth tests in liquid media without solvents as described by Klose and colleagues (27, 28). In contrast to mfe2Δ, loss of mfe2b slightly reduced growth on myristic and palmitic acids, but otherwise, the gene made little contribution. The mitochondrial β-oxidation gene had1 was also required for growth and filamentation, but had2 played a minor role.

In general, the signaling events leading to the filamentous growth response to fatty acids are not known, and we note that the ssp1Δ mutant, encoding the single homologue of a predicted dioxygenase important for oxylipin synthesis, was still able to switch to filamentous growth in response to a wide range of fatty acids (22) (data not shown). This result suggests that oxylipins, which influence mating and filamentation in other fungi, may not be the signal (7, 58). Overall, we hypothesize that intermediate metabolites or fatty acid derivatives generated by the β-oxidation process generate the signal for the switch to filamentous growth. We note that acetate is not able to provide the signal for filamentous growth.

Studies in several plant-pathogenic fungi reveal the importance of peroxisomal β-oxidation for disease initiation and development. For example, Colletotrichum species with defects in the assembly of peroxisomes (pex6 or pex13 deletions) were unable to grow on fatty acids and showed defects in appressorium formation and melanization leading to reduced glycerol levels (14, 26). Thus, the mutants were unable to penetrate the plant surface and form infectious hyphae. Deletion of the gene for isocitrate lyase, a key enzyme of the glyoxylate cycle in the peroxisomes of Colletotrichum lagenarium, led to an inability to utilize fatty acids and acetate (1). Interestingly, this mutant still made fully melanized appressoria but failed to produce infection hyphae. Connections between peroxisomes and infection are also seen in M. grisea, where pex6 mutants fail to melanize appressoria and to form infection hyphae (46). Similarly, deletion mutants of this fungus lacking carnitine acetyltransferase showed reduced melanization of appressoria and were nonpathogenic. These mutants did not grow on fatty acids or acetate, had a delayed mobilization of reserve lipids, and had a change in cell wall composition and structure (3, 46). A fox2/mfe2 mutant of M. grisea was also defective in appressorium formation and plant penetration (64). In A. alternata, a pex6 mutant was unable to grow on fatty acids, it was nonpathogenic, and it did not produce AK toxin (25). Finally, the expression of a peroxisomal 3-ketoacyl-CoA thiolase was also important for virulence in L. maculans (12). All these studies reveal the importance of peroxisomal function and β-oxidation for fungal phytopathogenicity. The results for U. maydis from our study further support the importance of peroxisomal β-oxidation and extend the analysis to demonstrate for the first time the importance of mitochondrial β-oxidation. U. maydis does make appressoria, so future studies should investigate the potential contribution of β-oxidation to the function of these penetration structures.

The importance of β-oxidation for disease development or virulence elaboration in animal and human pathogens such as C. albicans and C. neoformans has also been investigated. As in S. cerevisiae, C. albicans lacks mitochondrial β-oxidation, and both Piekarska et al. (43) and Ramirez and Lorenz (44) showed that a fox2 mutant did not grow on fatty acids or acetate and had reduced virulence. However, pex5 mutants of C. albicans did not show a reduced virulence and grew well on alternative carbon sources such as acetate or ethanol. Lorenz and Fink (33) showed that a mutant defective in isocitrate lyase in the peroxisomal glyoxylate pathway is reduced in virulence. Thus, as mentioned above, Piekarska et al. (42) speculated that a fox2Δ/fox2A mutant has disturbed transport of acetyl-CoA or glyoxylate cycle products across the peroxisomal membrane. However, the Ctf1 transcription factor that controls expression of β-oxidation genes and that is important for growth of C. albicans on fatty acids does not show pleiotropic growth defects (e.g., on acetate), but it still shows vir-
ulence attenuation, similar to the fox2Δ/fox2Δ mutant (45). Thus, further analysis is needed to examine metabolic connections between β-oxidation/peroxisomal function, the glyoxylate pathway, and the use of nonfermentable carbon sources by C. albicans during infection. Similarly, the importance of β-oxidation for virulence is just now being investigated in C. neoformans, which, like U. maydis, is a basidiomyces pathogen with both the peroxisomal and mitochondrial pathways. In contrast to C. albicans, loss of isocitrate lyase and defects in pex genes do not reduce the virulence of C. neoformans in mice (24, 48). In addition, there is evidence that the glyoxylate pathway may function outside the peroxisome in C. neoformans and that the peroxisome may function in glucose utilization (24). In a study performed in parallel with the work reported here and described in the accompanying article (28a), we recently showed that loss of β-oxidation functions reduced the virulence of C. neoformans, thus revealing this similarity between basidiomyces pathogens of plants and animals.

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