Peroxisomal and Mitochondrial β-Oxidation Pathways Influence the Virulence of the Pathogenic Fungus Cryptococcus neoformans

Matthias Kretschmer, Joyce Wang, and James W. Kronstad

Michael Smith Laboratories, Department of Microbiology and Immunology, and Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, Canada

An understanding of the connections between metabolism and elaboration of virulence factors during host colonization by the human-pathogenic fungus Cryptococcus neoformans is important for developing antifungal therapies. Lipids are abundant in host tissues, and fungal pathogens in the phylum basidiomycota possess both peroxisomal and mitochondrial β-oxidation pathways to utilize this potential carbon source. In addition, lipids are important signaling molecules in both fungi and mammals. In this report, we demonstrate that defects in the peroxisomal and mitochondrial β-oxidation pathways influence the growth of C. neoformans on fatty acids as well as the virulence of the fungus in a mouse inhalation model of cryptococcosis. Disease attenuation may be due to the cumulative influence of altered carbon source acquisition or processing, interference with secretion, changes in cell wall integrity, and an observed defect in capsule production for the peroxisomal mutant. Altered capsule elaboration in the context of a β-oxidation defect was unexpected but is particularly important because this trait is a major virulence factor for C. neoformans. Additionally, analysis of mutants in the peroxisomal pathway revealed a growth-promoting activity for C. neoformans, and subsequent work identified oleic acid and biotin as candidates for such factors. Overall, this study reveals that β-oxidation influences virulence in C. neoformans by multiple mechanisms that likely include contributions to carbon source acquisition and virulence factor elaboration.

The basidiomycete fungus Cryptococcus neoformans causes meningocencephalitis in immunocompromised people (33, 34). Cryptococcal disease became prominent in parallel with the HIV/AIDS epidemic, and the global burden is currently estimated at 1 million cases per year, with ~600,000 deaths (54). The majority of these cases result from infections by C. neoformans, although the related species Cryptococcus gattii has recently emerged as a pathogen of immunocompetent hosts (4, 30). C. neoformans is a facultative intracellular parasite, and interactions with alveolar macrophages are important for disease progression (78). C. neoformans survives within macrophages and may use host lipids for intracellular proliferation and to modulate the host immune response (14, 82). In particular, transcription profiling revealed that cryptococcal genes for peroxisomal β-oxidation, fatty acid import, and lipid degradation are upregulated upon macrophage internalization and during pulmonary infection (17, 24). Thus, fatty acid metabolism may be important for the virulence of C. neoformans in mammals.

β-Oxidation of fatty acids is important for the utilization of storage lipids or exogenous fatty acids to generate acetyl-coenzyme A (acetyl-CoA) for central carbon metabolism (22). Most organisms have multiple enzymes for each of the four steps in β-oxidation to accommodate fatty acids of different chain length or saturation state (47). In mammals, β-oxidation occurs in both peroxisomes and mitochondria. The peroxisome is thought to be responsible for the oxidation of long-chain fatty acids, and the mitochondrion oxidizes short-chain fatty acids and also performs the final oxidation step (23, 79, 80). Fungal β-oxidation is not well characterized, and it was previously thought that fungi might have peroxisomal β-oxidation only because Saccharomyces cerevisiae lacks the enzymes for mitochondrial β-oxidation (73, 75). However, recent in silico surveys of the pathways encoded in more than 50 fungal genomes revealed that most fungi possess both mitochondrial and peroxisomal pathways (10, 69). Mitochondrial β-oxidation has also been convincingly demonstrated in the saprophytic ascomycete Aspergillus nidulans (26, 44, 45).

In this study, we analyzed the roles of the peroxisomal and mitochondrial β-oxidation pathways in the growth and virulence of C. neoformans. Notably, we found that loss of the multifunctional enzyme Mfe2 for the second and third steps in peroxisomal β-oxidation resulted in multiple phenotypes, including reduced growth on fatty acids as the sole carbon source, decreased production of the polysaccharide capsule that is the major virulence factor of C. neoformans, and a diminished ability of the fungus to spread to or colonize the brain. A role for mitochondrial β-oxidation in virulence was also found through characterization of a had1 mutant lacking hydroxyacyl-CoA dehydrogenase, the third step in the pathway. Surprisingly, growth in the presence of wild-type C. neoformans cells or bacteria rescued a proliferation defect of the peroxisomal β-oxidation mutant, indicating detoxification of a medium component or provision of a growth factor. Overall, this work enhances our appreciation of the contribution of β-oxidation to nutrient acquisition, virulence factor expression, and the virulence of C. neoformans.

MATERIALS AND METHODS

Mutant construction and growth conditions. C. neoformans mutants derived from the wild-type strain H99 (see Table S1 in the supplemental material) were grown in yeast peptone dextrose (YPD) broth or agar, yeast nitrogen base (YNB) broth or agar, and minimal medium (MM; 15 mM...
glucose, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 13 mM glycine, and 3.0 mM thiamine) (8). Low-iron medium (LIM) was also employed to induce capsule, and this medium contains 27.5 mM glucose, 20 mM HEPES, 2.3 mM K₂HPO₄, 1.7 mM CaCl₂, 0.325 mM MgSO₄, 22 mM NaHCO₃, and 37.8 mM asparagine, as well as the microelements copper sulfate, zinc sulfate, manganese chloride, magnesium sulfate, sodium molybdate, boracic acid, and thiamine (76). An overlap PCR strategy was used to delete the \textit{MFE} and \textit{HAD} genes, and the genotypes of the resulting mutants were confirmed by colony PCR and genomic hybridization (see Fig. S1 in the supplemental material) (11). Briefly, \(1 \text{ kb of }' \text{ upstream sequence (left arm) and } \sim 1 \text{ kb of }' \text{ downstream sequence (right arm) were amplified along with a selectable marker from a plasmid containing a 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. (72). Transformants were grown overnight on YPD with 1 M sorbitol and then transferred to YPD with 100 \mu g mL⁻¹ nourseothricin or 200 \mu g mL⁻¹ neomycin. Gel electrophoresis, restriction enzyme digestion, and Southern blot hybridization were performed using standard procedures (65). Primers and plasmids are listed in Tables S2 and S3 in the supplemental material, respectively.

The \textit{MFE2} and \textit{HAD} genes were identified from the genome sequence at the Broad Institute (http://www.broadinstitute.org/scientific-community/data). The \textit{MFE2} gene (CNAG_03408) was identified in a BLASTp search with the \textit{Ustilago maydis} Mfe2 protein (UM00150). The mitochondrial \(\beta\)-oxidation genes were identified with the human \textit{Had} protein (3-hydroxyacyl-CoA dehydrogenase; NCBI CA655281.1, GI 14835311). The two \textit{C. neoformans} homologs are \textit{CNAG_03408} (HAD1) and \textit{CNAG_03134} (HAD2).

To delete the \textit{MFE2} gene, the left and right arms of the deletion construct were amplified with primers CnMfe2_1 and CnMfe2_2 and primers CnMfe2_5 and CnMfe2_6, respectively. The nourseothricin-resistant marker was amplified with primers CnMfe2_3 and CnMfe2_4 from plasmid pCH233. The final construct was amplified with nested primers CnMfe2_7 and CnMfe2_6, resulting in a deletion construct of 3.3 kb. Complementation of the \textit{mfe2Δ} mutant with a 7.0-kb construct was not successful, and three independent single-deletion mutants and two independent \textit{mfe2Δ had1Δ} double mutants were employed instead.

To delete the \textit{HAD1} gene, the left and right arms of the deletion construct were amplified with primers CnHad1_1 and CnHad1_2 and primers CnHad1_5 and CnHad1_6, respectively. The neomycin resistance marker was amplified with primers CnHad1_3 and CnHad1_4 from plasmid pAFA1. The final construct was amplified with nested primers CnHad1_7 and CnHad1_8, resulting in a deletion construct of 3.6 kb. The \textit{had1Δ} mutation was complemented by amplifying the complete \textit{HAD1} gene and promoter region with primers CnHad1_1comp1 and CnHad1_2comp, along with the nourseothricin resistance marker amplified with CnHad1_3comp and CnHad1_4comp. The PCR fragments were combined by an overlap reaction, and the final construct was amplified with CnHad1_1compn and CnHad1_1compn, resulting in a construct of 5.4 kb.

\textbf{RNA extraction.} Fungal cells were grown overnight in 5 ml of YPD and washed once with sterile water, and \(1 \times 10^6\) cells were transferred to 5 ml of YNB supplemented with either glucose or caproic, myristic, oleic, or linoleic acid (all added to 1%, except to 0.1% for caproic acid). The cells were then grown at 30°C and 250 rpm for 6 h, \(5 \times 10^6\) cells were harvested and washed, and RNA was extracted with a Qiagen RNaseasy minikit (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 \textit{C. neoformans} \(\beta\)-oxidation genes \textit{in vivo}, cells were pregrown in YPD, transferred to LIM for 24 h, washed three times in phosphate-buffered saline (PBS), and inoculated by intranasal instillation in \textit{Acr} mice; subsequently, \(1 \times 10^6\) cells were retrieved from the lungs of mice by lavage at 0 h, 8 h, 24 h after infection, as described by Hu et al. (24). The cells were washed three times with ice-cold water and lyophilized. RNA was extracted with the Qiagen RNaseasy minikit.

\textbf{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 was used in each reaction mixture. 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. 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 \textit{C. neoformans}.

\textbf{Growth assays on fatty acids.} To quantitate growth on fatty acids, cells were washed once with sterile water and \(1 \times 10^6\) cells were added to 5 ml of YNB 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 a sole carbon source at a concentration of 1% for all supplements except butyric and caproic acids (0.01%) and lauric acid (0.1%). To investigate the accumulation of toxic \(\beta\)-oxidation intermediates, galactose, lactate, or acetate was added at 1%. 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. Cells were observed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany). Images were captured with a digital video camera and processed with Adobe Photoshop (version 7) software.

\textbf{Influence of \(\beta\)-oxidation on protease secretion and response to stress.} The following inhibitors and agents were added to YNB or YPD at the indicated concentrations to provoke stress: NaCl (1.2 M), caffeine (0.5 mg ml⁻¹), tert-buty1 hydroperoxide (25 µg ml⁻¹), brefeldin A (BFA; 250 µM), monensin (250 µM), fluconazole (5 µg ml⁻³ or 10 µg ml⁻³), Congo red (0.1%), and LiCl (100 mM). Exponentially growing cultures of \textit{C. neoformans} were washed with YNB, resuspended in the same medium, and adjusted to \(2 \times 10^6\) cells ml⁻¹. After 10-fold serial dilutions, \(5 \mu l\) of each dilution was spotted onto YPD or YNB plates containing different stress agents. The plates were incubated for 2 to 4 days at 30°C or 37°C and photographed. Extracellular protease activity was assessed on agar plates containing 1.5% agar, 15 mM glucose, 13 mM glycine, 29.4 mM KH₂PO₄, 10 mM MgSO₄, 3 µM thiamine, and 0.1% azaobulin (pH 4.5) as described by Chen et al. (6). The plates were inoculated with \(10^6\) cells in 5 µl. After incubation for 14 days at 30°C, the plates were photographed to assess extracellular protease activity by halo size surrounding the colonies. Ergosterol sensitivity was tested on YPD plates with 100 µg ml⁻¹ ergosterol. Sensitivity against ethanol (EtOH) was tested in a 96-well plate with 100 µl YPD with 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.

\textbf{Assays with conditioned media.} To assess the ability of wild-type \textit{C. neoformans} cells to produce a growth-promoting factor for the \textit{mfe2Δ} mutant, a YPD plate was spread with \(\sim 750\) cells of the \textit{mfe2Δ} mutant and a 5-µl droplet with \(10^6\) wild-type cells was spotted in the center of the plate. Spot assays with 10-fold serial dilutions were also used on YPD agar. Plates were incubated at 30°C for up to 3 days. Conditioned liquid medium to test the growth-promoting activity of the wild-type strains H99 and JEC21 and a mutant defective in producing the quorum-sensing peptide (\textit{appLΔ}) was obtained from 25-ml YPD cultures grown for 24 h. The cultures were centrifuged, and the supernatant was filtered sterilized. The activity of these supernatants was tested at volumes of 2.5 ml, 1.5 ml, 0.5 ml, and 0 ml of conditioned medium plus fresh YPD to reach a final volume of 5 ml. The media were inoculated with \(10^6\) of washed cells ml⁻¹ and incubated for 36 h at 30°C with shaking. Cell numbers were determined with a hemacytometer, and the culture tubes were photographed. Conditioned medium was fractionated with spin columns with molecular mass exclusions of 100 kDa, 50 kDa, and 10 kDa. The fractionated condi-
tioned medium (1.5 ml) was added to 3.5 ml of fresh YPD, and 10^4 washed mfe2Δ cells ml^-1 were added. Cell numbers were determined after incubation for 36 h at 30°C. For heat treatment, 2 ml of filter-sterilized H99 supernatant was incubated for 5 min at 50°C, 75°C, or 100°C. The treated medium (1.5 ml) was added to 3.5 ml fresh YPD with 10^4 washed mfe2Δ cells ml^-1. For plate assays with oleic acid or linoleic acid, 1 μl of filter-sterilized fatty acid was pipetted in the center of a YPD plate inoculated with ~750 cells of the mfe2Δ mutant. To test the rescue activity of bacteria and fungi, 5 μl overnight cultures was pipetted in the center of the YPD plate. The plates were incubated for 36 h at 30°C and photographed.

**Virulence assay.** The virulence of C. neoformans strains was examined in C57Bl/6 mice by intranasal inoculation with 5 × 10^5 cells in a total volume of 50 μl PBS. The fungal load in the lungs and brains of infected animals was determined by plating homogenized tissue and counting the CFU. All experiments with vertebrate animals were conducted in full compliance with the guidelines of the Canadian Council on Animal Care and the University of British Columbia’s Committee on Animal Care. The studies involving mice were approved by the University of British Columbia’s Committee on Animal Care (protocol A08-0586).

**Phenotypic assays.** Capsule formation of C. neoformans was examined by differential interference microscopy (DIC) after incubation for 24 h and 48 h at 30°C in LIM, minimal medium, or modified LIM and staining with India ink. Melanin production was examined on L-3,4-dihydroxyphenylalanine (L-DOPA) plates containing 0.1% glucose (25).

**Statistical analysis.** The data are representative of at least three independent experiments. Values are given as the mean of triplicates ± standard deviation. The virulence data were analyzed with the log rank test for statistical differences.

**RESULTS**

**Identification of genes encoding β-oxidation enzymes in C. neoformans.** Defects in β-oxidation compromise virulence in phytopathogenic fungi (2, 31, 32, 61, 81; see also the accompanying article by Kretschmer et al. [33a]). For example, we previously demonstrated that a mutant lacking the multifunctional enzyme Mfe2 in the basidiomycete *Ustilago maydis* had reduced virulence and sporulation on maize (32). In addition, the loss of β-oxidation in the human pathogen *Candida albicans* slightly attenuates virulence, although this fungus has only the peroxisomal pathway (55, 56, 59, 60). Given that C. neoformans possesses both mitochondrial and peroxisomal β-oxidation, we hypothesized that these functions might contribute to virulence. We therefore inspected the genome of C. neoformans (the serotype A strain H99) and identified a single candidate MFE2 gene for the multifunctional enzyme in the peroxisomal pathway as well as two genes, HAD1 and HAD2, encoding the 3-OH-acyl-CoA dehydrogenase enzyme for the mitochondrial pathway. The organelle location of the proteins is deduced from the sequence homology of known proteins and their locations and from predicted organelle import sequences of the proteins.

We examined MFE2 more closely and found the closest homologues for this gene in the basidiomycetes *Laccaria bicolor*, *U. maydis*, and *Puccinia graminis* (see Table S4 and Fig. S2 in the supplemental material). Mfe2 has a predicted peroxisomal PTS1 signal peptide with the sequence AKL (http://www.peroxisomedb.org/Target_signal.php). The closest homologues of the HAD1 gene were from *U. maydis*, *Malassezia globosa*, and *Dano rerio*, while the closest homologues for HAD2 were from *L. bicolor*, *Glomerella graminicola*, and *Schizopyllum commune* (see Table S4 and Fig. S3 in the supplemental material). Had1 has a predicted mitochondrial targeting sequence of 35 amino acids (P = 0.9829), while Had2 has a sequence of 52 amino acids (P = 0.6508), according to MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html). Homologues of HAD2 from *C. neoformans* were also identified in ascomycetes and bacteria. Interestingly, the HAD1 gene clustered with the human HAD1 gene in one clade, while the HAD2 gene was in a second clade (see Fig. S3 in the supplemental material). In this study, we focused on MFE2 in the peroxisomal pathway because only a single gene was identified in the genome and we could therefore avoid issues arising from redundancy. We also focused on HAD1 because the ortholog of this gene made the primary contribution to mitochondrial β-oxidation in *U. maydis* (see the accompanying article [33a]). Indeed, as described below, deletion analysis revealed that loss of HAD1 in *C. neoformans* caused phenotypes comparable to those from the loss of HAD1 in *U. maydis*. We also targeted the HAD1-encoded 3-OH-acyl-CoA dehydrogenase step in the mitochondrial β-oxidation pathway because loss of this enzyme caused the accumulation of inhibitory intermediates in *A. nidulans* (26, 44, 45). We reasoned that this might also reduce virulence for *C. neoformans* and therefore provide an additional incentive to target fungal β-oxidation as an antifungal strategy.

**Fatty acid induction and glucose repression of β-oxidation genes.** We initially examined expression of MFE2, HAD1, and HAD2 in cells grown in culture and found that these genes were upregulated in the presence of exogenous fatty acids, relative to growth in glucose (Fig. 1A). Induction occurred regardless of the chain length or saturation state of the fatty acids. In addition, the transcripts of MFE2 were generally more highly induced than those of the mitochondrial genes (Fig. 1A). β-Oxidation facilitates lipid utilization in the absence of glucose and other preferred carbon sources, and glucose represses the assembly of the peroxisome and the expression of β-oxidation genes in some fungi (13, 19). To examine glucose repression, strains were grown for 2 days with myristic acid and then transferred to fresh medium with myristic acid alone, myristic acid and glucose, or glucose alone. Relative to myristic acid alone, growth in the presence of myristic acid and glucose or glucose alone resulted in a repression of the peroxisomal and mitochondrial β-oxidation genes (Fig. 1B). In this experiment, the decrease in transcript levels ranged from 2.9-fold for HAD2 to 20-fold for MFE2, thus supporting the conclusion that glucose repression occurs for these genes in *C. neoformans*.

**Expression of β-oxidation genes during infection.** Alternative carbon sources such as fatty acids may be important during infection and may be particularly relevant for the proliferation of intracellular pathogens following phagocytosis by macrophages (17, 24, 43, 71). We therefore examined the expression of β-oxidation genes during lung infection of mice by *C. neoformans* (Fig. 1C). The MFE2, HAD1, and HAD2 genes each showed elevated transcript levels at 8 h and 24 h during murine infection relative to those in cells grown in a low-iron medium with glucose (LIM) that induces capsule formation (Fig. 1C) (76). For example, the transcript level for HAD1 was 3.5-fold higher at 8 h and the transcript level for MFE2 was 6.1-fold higher at 24 h. The transcript levels of the genes were similar between the 8-h and 24-h samples. Surprisingly, the β-oxidation genes were highly expressed when cells were grown in the rich medium YPD used to prepare the cells for mouse inoculation compared to their level of expression in minimal low-iron medium with glucose that is typically used to induce capsule expression (Fig. 1C). We employed YPD because it contains 2% glucose, whereas LIM contains 0.5% glucose, and we assumed that the expression of the β-oxidation genes would be repressed. How-
genes are as follows: MFE2, HAD1, and HAD2. Cells were grown in YNB 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. (B) Quantitative PCR analysis of glucose repression of transcript levels for \( \beta \)-oxidation genes. Cells were pre-grown for 48 h in YNB with 1% myristic acid as the carbon source, washed in water, and transferred to YNB with either 1% myristic acid alone, myristic acid and glucose, or glucose alone for 6 h. (C) Quantitative PCR analysis of transcript levels for \( \beta \)-oxidation genes in a murine mouse model of cryptococcosis. RNA was isolated at 0 h (LIM or YPD), 8 h, and 24 h for \( C.\ neoformans \) infection stages. 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.

However, the unexpected discovery of expression in YPD suggests that the genes are subject to other regulatory influences in YPD that compensate for or overcome glucose repression. Overall, these results indicate that the transcripts of the \( \beta \)-oxidation genes in \( C.\ neoformans \) were expressed during infection and that their mRNA levels are elevated relative to those under the specific in vitro growth condition of LIM.

Deletion of \( \beta \)-oxidation genes influences the growth of \( C.\ neoformans \) on fatty acids and acetate and leads to the accumulation of toxic intermediates. We next constructed mutants lacking MFE2 or HAD1 to examine the contributions of the peroxisomal and mitochondrial pathways to growth on fatty acids, virulence-related phenotypes, and virulence (see Fig. S1 in the supplemental material). As shown in Fig. 2A, the wild-type strain utilized a broad range of fatty acids for growth (albeit growth was reduced relative to that with glucose), including short-chain (C4 to C6), medium-chain (C12), long-chain (C14 to C16), and polyunsaturated, long-chain (e.g., C18:2) fatty acids. Overall, the wild-type cells grew best on myristic and lauric acids, while the short-chain fatty acids butyric acid (C4:0) and caproic acid (C6:0) appeared to be toxic at 1%, although a lower concentration (0.01%) supported weak growth. The medium-chain fatty acid lauric acid (C12:0) also showed toxicity at a concentration of 1%, but growth was supported at a lower concentration (0.1%). The three independent \( mfe2D \) mutants had growth defects on fatty acids of all chain lengths and saturation, with the exception that weak growth was observed on butyric acid. The \( had1D \) mutant also grew poorly on butyric, caproic, lauric, and linoleic acids, and wild-type levels of growth were observed for the complemented mutant on these fatty acids. Importantly, the \( had1D \) mutant showed growth similar to that of the wild-type strain on myristic, palmitic, and oleic acids. The mutant lacking both MFE2 and HAD1 failed to grow on any of the fatty acids, thus supporting the idea that loss of these genes results in a block of both the mitochondrial and peroxisomal \( \beta \)-oxidation pathways. We noted that both pathways are important for utilization of the polyunsaturated long-chain fatty acid linoleic acid (C18:2), as well as caproic and lauric acids (Fig. 2A).

To test the possibility that defects in \( \beta \)-oxidation caused accumulation of toxic intermediates as seen in other fungi, the wild-type strain and the mutants were grown on the alternative carbon sources galactose, lactose, and acetate (16, 26, 40, 44, 45, 48). No growth was observed on lactose, and galactose generally behaved like glucose as a catabolite-repressing carbon source (Fig. 1B and 2B). That is, the mutants grew on fatty acids in the presence of galactose probably because of the repression of \( \beta \)-oxidation, although lauric acid did partially inhibit the \( mfe2D \) mutant in the presence of galactose (Fig. 2B). In contrast, acetate is a nonrepressing carbon source for \( C.\ neoformans \), although the \( mfe2D \) mutant does not grow as well as the wild-type strain on acetate in either liquid (Fig. 2C) or solid medium (Fig. 3). We found that the combination of acetate plus lauric acid led to poor growth for all of the strains (wild-type, \( mfe2D \), and \( had1D \) strains) compared to the growth on acetate alone. Additionally, the poor growth of the \( mfe2D \) mutant on oleic acid was not improved when acetate was included in the medium (Fig. 2C). Acetate also improved the growth of the \( had1D \) mutant on oleic acid but not on lauric acid (Fig. 2C). These results suggest that toxic intermediates may accumulate during growth of all of the strains on the medium-chain fatty acid lauric acid and during growth on oleic acid for the \( mfe2D \) mutant. Taken together, it appears that a block in peroxisomal or mitochondrial \( \beta \)-oxidation may lead to the accumulation of toxic intermediates.

We also examined the growth of the mutants on solid medium containing acetate (C2), lactate (C3), glycerol (C3), arabinose (C5), or citrate (C6) and found that the \( mfe2D \) mutant showed weak growth on acetate (as mentioned above). The HAD1 gene was not required for growth on this carbon source, and the \( mfe2D \) \( had1D \) double mutant behaved like the \( mfe2D \) mutant (Fig. 2C and 3). No differences for the other alternative carbon sources were found (Fig. 3; data not shown). Overall, these results suggest a connection between acetate utilization and \( \beta \)-oxidation, as identified in some fungi such as \( Candida albicans \) (35, 36). However, we noted that the three \( mfe2D \) mutants and the \( mfe2D \) \( had1D \) double mu-
tant also showed less robust growth on the complex medium YPD and on YNB (although it was better than that on acetate). Interestingly, the mutant that was closest to the wild-type strain appeared to grow better, suggesting the presence of a diffusible growth factor (Fig. 3, YPD plate). This observation is examined in more detail below.

A defect in peroxisomal β-oxidation influences the stress response and extracellular protease activity. As indicated above, the inability of cells to catabolize fatty acids can lead to the accumulation of toxic fatty acids or their metabolites (16, 26, 40, 44, 45, 48). In addition, a defect in β-oxidation can alter the lipid composition of the plasma membrane due to the absence of specific fatty acids and phospholipids (42). These changes could influence trafficking of materials to the cell wall and the extracellular milieu and impair the ability of β-oxidation mutants to respond to and survive environmental stress. We therefore tested the growth of the three mfe2Δ mutants, the had1Δ and mfe2Δ had1Δ mutants, and, in some assays, the had1Δ HAD1-complemented strain under a variety of stress conditions, including growth at 37°C and growth in the presence of NaCl, LiCl, fluconazole, tert-butyl hydroperoxide, monensin, BFA, caffeine, Congo red, EtOH, and ergosterol (Fig. 4A to D and data not shown). Growth on YPD or YNB medium without additions served as controls. In these assays, the mfe2Δ mutants displayed increased susceptibility to agents that challenge cell wall integrity (e.g., caffeine and Congo red), as well as the antifungal drug fluconazole (Fig. 4C). Fluconazole inhibits Erg11 in the ergosterol biosynthesis pathway, and we noted that the mfe2Δ mutants also showed diminished growth on ergosterol, further suggesting that the pathway is perturbed (Fig. 4D). A slight growth defect was also seen for the mfe2Δ mutants on YNB minimal medium compared to the growth for the wild-type strain and the had1Δ mutant (Fig. 4C). This phenotype was noted above, as was the more surprising compromised growth of the mfe2Δ mutants on the complete medium YPD (Fig. 3 and 4D). Therefore, the peroxisomal β-oxidation pathway is important for...
growth on a complex medium, and this observation may be related to our observation that the MFE2 transcript is elevated in cells grown on YPD (Fig. 1C). No differences were observed between any of the mutants and the wild-type parental strain for growth at 37°C or in the presence of the salts, oxidative stress agents, or the secretion inhibitor BFA (data not shown). Additionally, all strains were able to grow in medium containing up to 6% EtOH (data not shown).

As shown in Fig. 4C, we also observed that the mfe2Δ mutants were sensitive to the secretion inhibitor monensin. We therefore tested additional phenotypes that are dependent on intracellular trafficking to further examine the possibility that secretion is altered in the β-oxidation mutants. Melanin production is dependent on trafficking of laccase to the cell wall with concomitant copper loading, and we found that melanin production was reduced in the mfe2Δ mutants on L-DOPA medium, although the mutants also displayed slower growth (Fig. 4B). Additionally, we examined the level of extracellular protease activity on solid medium containing azoalbumin (6). As shown in Fig. 4A, the mfe2Δ mutants and the mfe2Δ had1Δ mutant in particular showed elevated extracellular protease activity compared to the wild-type strain. Notably, the mfe2Δ mutants also had a reduced capsule size, and this phenotype is further analyzed below. Overall, these results indicate that loss of β-oxidation causes several phenotypic changes in C. neoformans that may be generally related to secretion and the response to cell wall stress. This is consistent with possible changes in fatty acid and phospholipid levels, perhaps in trafficking vesicles, as a result of a defect in peroxisomal β-oxidation. It was striking, however, that loss of the HAD1 gene for mitochondrial β-oxidation did not influence stress or secretion, although HAD2 might have a compensatory function in this case.

Peroxisomal β-oxidation influences capsule formation. C. neoformans cells are induced to produce a large polysaccharide capsule when grown in LIM (76). The carbon source in LIM is important because we found that wild-type cells failed to produce a capsule when grown in LIM with fatty acids instead of glucose (Fig. 5A). We noted that capsule was not induced at any time during growth in LIM with fatty acids, even with prolonged incubation (Fig. 5A). This result suggests that the production of acetyl-CoA via β-oxidation and subsequent gluconeogenesis to generate precursors is not sufficient to support capsule elaboration. In contrast, acetate does support capsule formation when provided as the sole carbon source (M. Kretschmer, E. Griffith, and J. W. Kronstad, unpublished results). In further support of a role for β-oxidation, we found that, in contrast to wild-type cells, deletion of MFE2 (but not HAD1) reduced capsule formation in LIM with glucose (Fig. 5B). To further examine the capsule defect, we grew the mfe2Δ mutant in a variety of media and found that MM did support capsule production, although considerable variation in size was observed (Fig. 5). A comparison of the compositions of LIM and MM suggested that differences in magnesium levels (31 times higher in MM), phosphate levels (13 times higher in MM),
and nitrogen source (13 mM glycine in MM versus 37.8 mM asparagine in LIM) might account for differences in capsule size (Fig. 5C and D). We tested these components and found that addition of phosphate to LIM, but not manganese sulfate, led to capsule formation for the mutant. In addition, elimination of asparagine as the nitrogen source or replacement with glycine resulted in capsule induction for the \textit{mfe2}/H9004 mutant in LIM (Fig. 5C). We hypothesize that loss of peroxisomal \textit{\textbeta}-oxidation caused an alteration in carbon metabolism leading to a capsule defect and that phosphate and nitrogen availability contributed to the medium effects on capsule size. Certainly, a link between capsule and nitrogen metabolism in \textit{C. neoformans} has been established (39), and extensive work in \textit{S. cerevisiae} illustrates the interconnections between carbon and nitrogen metabolism (20, 84).

**Conditioned medium, oleic acid, and biotin stimulate the growth of the \textit{mfe2}\textDelta mutant.** As mentioned above, during our analysis of the growth of the \textit{mfe2}\textDelta mutants on solid medium, we observed that the strains formed larger colonies when grown in proximity to the wild-type strain, suggesting the presence of an exported growth factor or depletion of an inhibitory factor (Fig. 3, 4D, and 6A). Improved growth of the \textit{mfe2}\textDelta mutants was visually apparent in liquid YPD medium that was conditioned by the growth of wild-type cells (Fig. 6B). In addition, cell counts revealed that the growth of the \textit{mfe2}\textDelta mutant was improved \ (~3-fold, \sim 79-fold, and \sim 197-fold when as little as 10\%, 33\%, or 50\% of the medium, respectively, was replaced with the medium conditioned by the wild-type strain (Fig. 6B and its legend). We initially considered the possibility that stimulation was due to the known quorum-sensing peptide encoded by the \textit{QSP1} gene in \textit{C. neoformans} because this peptide stimulates growth (38). However, YPD conditioned by growth of the \textit{qsp1}\textDelta deletion mutant still rescued the growth defect of the \textit{mfe2}\textDelta mutant, as did medium from the wild-type parental strain JEC21 (Fig. 6B and its legend).

The wild-type strain also provided the rescue activity for the \textit{mfe2}/H9004 \textit{had1}/H9004 double mutant, and the \textit{had1}/H9004 mutant rescued the \textit{mfe2}/H9004 growth defect, while the \textit{mfe2}/H9004 \textit{had1}/H9004 double mutant could not rescue the \textit{mfe2}\textDelta phenotype (data not shown). Addition of the conditioned medium to H99 cells had no effect on the cell number independent of the volume or the origin of the conditioned medium (data not shown).

As a first step toward identifying the factor(s) that influenced growth, size-fractionated conditioned medium was tested for activity with the \textit{mfe2}\textDelta mutant. The fractions below 10,000 Da for the wild-type and \textit{qsp1}\textDelta strains had activity, thus indicating the

**FIG 5** Peroxisomal \textit{\textbeta}-oxidation is required for capsule formation in \textit{C. neoformans}. (A) Fatty acids as the sole carbon source do not induce capsule formation in strain H99 after prolonged incubation. H99 cells were incubated in LIM with 0.5% glucose or myristic, palmitic, oleic, or linoleic acid, 0.01% caproic acid, or 0.1% lauric acid for 7 days at 30°C. Cells were stained with India ink to reveal the extracellular capsule. (B) The wild-type, mutant, and complemented strains were incubated for 48 h in capsule-inducing medium (LIM) and stained with India ink to reveal the extracellular capsule. (C) The \textit{mfe2}\textDelta mutant and the wild-type strain were grown in MM and modified LIM to assess capsule formation by India ink staining. LIM was supplemented with 10 mM MgSO\textsubscript{4} or 29.4 mM K\textsubscript{2}HPO\textsubscript{4}. LIM without asparagine was also employed with or without 13 mM glycine. (D) India ink stains of wild-type cells of \textit{C. neoformans} strain H99 grown in MM (containing glucose) with no addition or with 0.02% oleic or linoleic acid at 30°C for 48 h. Representative examples of cells are shown.
involvement of a small molecule or protein (data not shown). Conditioned medium was also treated for 5 min at an elevated temperature (50°C, 75°C, or 100°C), but no loss of activity was shown, suggesting that a heat-labile protein was not responsible (Fig. 6C). Next, a variety of small molecules were tested as candidates for the growth factor, including phosphatidylcholine, which was recently found to induce capsule formation, as well as inositol and farnesol, which act as signaling molecules in fungi (8, 37, 83). However, no influence on mutant growth was found (data not shown). Because the mfe2Δ growth phenotype resembled the petite phenotype of S. cerevisiae mutants with defects in the ergosterol pathway, we also tested ergosterol, cholesterol, methionine, hemin, and different unsaturated fatty acids, including oleic and linoleic acids, for activity (18, 29). Of these, only low concentra-
tions of oleic acid in YPD improved growth, while linoleic acid reduced the growth of the mfe2Δ mutant and the wild-type strain (Fig. 6D to F). As mentioned earlier, we found that ergosterol mildly inhibited the growth of the mutant (Fig. 4). Gram-positive and Gram-negative bacteria, but not another basidiomycete fungus (U. maydis), also improved the growth of the mfe2Δ mutant, and the response was more robust than that with the wild-type C. neoformans strain or oleic acid (Fig. 6D). We examined the influence of oleic and linoleic acid in more detail and found that linoleic acid resulted in a zone of improved growth when spotted on a plate with the mfe2Δ mutant, perhaps indicating that an optimum concentration or a modification was needed or that impurities in the linoleic acid influenced the activity (Fig. 6D).

We noted that inclusion of 0.02% oleic acid in YPD that also contains 2% glucose led to slightly better growth after 24 h of both the wild-type strain and the mutant, while inclusion of 0.02% linoleic acid reduced growth (Fig. 6E and F). We also examined the activities of oleic and linoleic acid on wild-type cells during growth in MM with glucose, which should repress β-oxidation and therefore not cause the accumulation of toxic β-oxidation intermediates (Fig. 1B and 2B). Oleic acid increased the growth rate of the wild-type strain by a factor of 3.0 (± 0.5) in MM with 0.02% oleic acid and that of the mfe2Δ mutant by a factor of 7.0 (± 1.8) in YPD after 24 h. This suggested a more general role for the fatty acid as a signaling factor rather than a carbon source (Fig. 6E). However, the growth-promoting activity of oleic acid for the mfe2Δ mutant in YPD occurred at oleic acid concentrations 1,000 times lower than the concentration for the wild-type strain, thus indicating greater sensitivity. Surprisingly, 0.02% linoleic acid abolished (20 times less) the growth of the wild-type strain in MM with glucose (Fig. 6F). In this case, we would have expected glucose to repress β-oxidation and not allow the accumulation of toxic β-oxidation intermediates (Fig. 1B, 2B, and 5F). This result therefore suggests that linoleic acid has signaling activity opposite that of oleic acid. No differences in cell shape, size, or capsule formation were observed between wild-type cells cultured in MM with or without 0.02% oleic acid. However, cells cultured in MM with 0.02% linoleic acid showed poor capsule formation (Fig. 5D).

Finally, we also considered biotin to be a rescue factor for the mfe2Δ mutant because the biotin biosynthetic pathway in fungi might be located in the peroxisome and its precursor (pimelic acid) might depend on β-oxidation activity (46). In a spot test, we found that biotin also showed a weak rescue activity for the mfe2Δ mutant (Fig. 6G).

Overall, these data suggest that oleic acid may be a growth factor for C. neoformans, that this fatty acid and/or additional growth factors such as biotin can be provided by bacteria, and that linoleic acid has more complex positive and negative influences on both growth and capsule formation. Whether the growth stimulation of the mfe2Δ mutant observed with oleic acid is related to the conditioned medium influence or if additional stimulatory or inhibitory factors are involved remains to be determined.

β-Oxidation is required for virulence in C. neoformans. The β-oxidation genes were expressed during infection, suggesting that fatty acid catabolism may be important for growth in the host (Fig. 1C). In addition, loss of MFE2 resulted in a defect in capsule formation, a major virulence factor in C. neoformans. Therefore, we tested the virulence of the C. neoformans mutants in a murine inhalation model of cryptococcosis (Fig. 7). An initial pilot study with three mice per strain revealed that the had1Δ mutant showed virulence similar to that of the wild-type strain and that the mfe2Δ mutants and an mfe2Δ had1Δ mutant had attenuated virulence (Fig. 7A). A subsequent larger-scale assay confirmed that the mice infected with the wild-type strain and the had1Δ mutant showed no difference in survival (~day 20). In contrast, the three independent mfe2Δ mutants were hypovirulent and the mice survived 8 to 10 days longer (Fig. 7B). This result indicates that the peroxisomal pathway may play a greater role in virulence than the mitochondrial pathway. However, the two double mutants lacking MFE2 and HAD1 showed further attenuation of virulence and the infected mice survived until day 43 to 45 (Fig. 7B). Therefore, HAD1 made a contribution to virulence that was apparent in the background of the mfe2Δ mutation.

The virulence of the C. neoformans mutants was examined in more detail by measuring the fungal burden in the lungs and brains of the infected mice (Fig. 8). For both the pilot study (Fig. 8A) and the larger assay (Fig. 8B), the numbers of CFU in the lungs of mice were similar at the day of sacrifice for the mutants and the wild-type strain. In contrast, the fungal burden in the brain was 4

Fig 7 β-Oxidation is required for full virulence of C. neoformans. The virulence of C. neoformans β-oxidation mutants was analyzed in a murine inhalation model of cryptococcosis. (A) Results of a pilot study in which three mice were inoculated with 5 × 10⁵ cells of the wild-type strain, the had1Δ mutant, two independent mfe2Δ mutants, and one mfe2Δ had1Δ mutant. The mice were sacrificed at a weight loss of 15%. The mfe2Δ and mfe2Δ had1Δ groups are significantly different from wild type (wt) (P < 0.05), while had1Δ is not different from wild type, according to a log rank test. (B) A larger experiment with 10 mice (with the exception of the mfe2Δ had1Δ mutant, where one mouse died during the inoculation process). The inoculated strains were the wild-type strain, the had1Δ mutant, three independent mfe2Δ mutants, and two mfe2Δ had1Δ mutants. The had1Δ HAD1 strain was not included because of lack of a virulence defect for the had1Δ mutant in the pilot study. Three independent mfe2Δ mutants were used. The results for the mfe2Δ and mfe2Δ had1Δ groups are significantly different from those for wild type (P < 0.001), while the result for the had1Δ mutant is not different from that for wild type, according to a log rank test.
The mfe2Δ mutants display a reduced fungal load in the brain but not in the lung. Fungal burden was analyzed with three mice at the time of sacrifice for the virulence assays whose results are shown in Fig. 7A and B. The mice were sacrificed at a weight loss of 15%. (A) Results of the pilot study; (B) results of the main study. The horizontal line in each graph shows the detection limit of the assay. Standard deviations are shown.

DISCUSSION

In this study, we demonstrated that genes in both the peroxisomal and mitochondrial β-oxidation pathways contribute to fatty acid utilization, virulence-related phenotypes, and virulence in *C. neoformans*. The transcripts for the genes in these pathways were expressed during infection, induced by fatty acids in culture, and repressed by glucose under some medium conditions. In addition, the growth phenotypes of the β-oxidation mutants were consistent with mitochondrial processing of short-chain fatty acids and peroxisomal activity with long-chain fatty acids, with final oxidation taking place in the mitochondria. Similar organelle contributions are observed in mammalian cells and *A. nidulans* (44, 45, 62, 63, 79). We did observe that linoleic acid (C18:2) utilization required both mitochondrial and peroxisomal β-oxidation. This result is consistent with observations on mitochondrial β-oxidation of linoleic acid in mammalian cells and the partnership of the two pathways to accomplish full growth on long-chain and very-long-chain fatty acids in *A. nidulans* (9, 44).

The mfe2Δ mutants showed a variety of phenotypes beyond those directly related to defects in growth on fatty acids. These included reduced capsule and melanin production and increased susceptibility to agents that challenge cell wall integrity, the antifungal drug fluconazole, the secretion inhibitor monensin, and ergosterol. A potential impact on secretion was also evident from the increased extracellular protease activity observed for the mfe2Δ and mfe2Δ had1Δ mutants. Proteases could potentially contribute to virulence, and *C. neoformans* is known to secrete a variety of proteases (6, 12). In general, the pleiotropic phenotypes may be due to several possible metabolic perturbations, including the accumulation of intermediates (e.g., acyl-CoA molecules), altered fatty acid availability leading to changes in phospholipid and sterol composition in membranes, and reduced acetyl-CoA production (42). The small capsule size could also be indirectly due to metabolic changes in carbon and nitrogen metabolism, reduced synthesis and/or trafficking of capsule material, and/or changes in the cell wall structure leading to inefficient capsule attachment (28, 39, 53). Although many factors regulate capsule elaboration, it is known that a defect in acetylation of the polysaccharide can influence capsule size, and it is possible that reduced acetyl-CoA levels due to a block in β-oxidation may reduce acetylation (28, 35).

The observed attenuation in virulence for the mfe2Δ mutant is consistent with the pleiotropic influence of the mutation on virulence factor elaboration and growth on fatty acids. Virulence was further attenuated upon deletion of both MFE2 and HAD1, thus indicating contributions of both pathways during infection. We hypothesize that the reduced virulence of the mutants is due to a requirement for fatty acids as a carbon source during intracellular parasitism in phagocytic cells or in specific tissues, plus pleiotropic influences of β-oxidation on other metabolic functions, such as growth on acetate as well as capsule and melanin production. The attenuated virulence and low fungal burden in the brain for the *C. neoformans* mutants can be compared with the findings for peroxisomal β-oxidation and virulence in *C. albicans* (55, 56). In this fungus, a fox2Δ/fox2Δ mutant lacking the second enzyme in the peroxisomal pathway had reduced virulence, although a defect in brain colonization was not observed (in contrast to the situation in *C. neoformans*). Similarly, the transcription factor Cft1 regulates β-oxidation and participates in virulence to a similar extent as Fox2 (60). However, Cft1 does not appear to influence growth on acetate and thus likely does not interfere with the glyoxylate pathway (60). The situation in *C. albicans* is complex because of interactions between peroxisomal function and the glyoxylate pathway (36, 55, 56, 59, 60). This pathway is important in the host because *C. albicans* mutants lacking the glyoxylate enzyme isocitrate lyase or malate synthase are attenuated for virulence and fail to grow on alternative carbon sources such as acetate (43). The glyoxylate pathway provides the means to convert C3 compounds to C4 precursors for biosynthesis, allowing growth on fatty acids and C2 compounds such as acetate. Thus, the operation of the glyoxylate pathway requires transport of several intermediates across the peroxisomal membrane and depends on mitochondrial metabolism (36, 49). An understanding of the operation and regulation of the glyoxylate cycle and its integration with cellular metabolism will require further investigation of the participating metabolite transporters in the peroxisomal membrane. *C. neoformans* is clearly different in its metabolic capabilities because mutants lacking isocitrate lyase or malate synthase also fail to grow on acetate but retain full virulence (27, 64). A difference for *C. neoformans* is also illustrated by the finding that pex1Δ and pex6Δ mutants with defects in peroxisomal function grew poorly on glucose, leading to speculation that peroxisomes might function in glycolysis or the regulation of glucose utilization (27). However, *C. neoformans* mutants that are impaired in glucose utilization are attenuated for...
virulence and show decreased persistence in the central nervous systems of rabbits and mice (57).

The mfe2Δ mutants in C. neoformans showed reduced growth on the complex medium YPD, and this phenotype could be rescued by conditioned medium and exudates from wild-type cells and bacteria. Although a more detailed investigation is needed, we favor the involvement of a growth-promoting factor rather than detoxification of an inhibitor, for two reasons. First, it is likely that an enzymatic detoxification activity would have been inactivated by heat treatment. Second, the diffusion assay with a central colony of the wild-type strains of bacteria appears to be more consistent with an easily diffusible growth factor rather than a detoxification enzyme. However, it is possible that detoxification could occur by a nonenzymatic mechanism. Additional work is needed to identify potential inhibitors in YPD as well as growth-promoting activities, although we did find that oleic acid and biotin, which depends on β-oxidation activity during its synthesis, could function in this capacity for the mfe2Δ mutant and the wild-type strain, while linoleic acid inhibited the growth of both strains. We therefore hypothesize that C. neoformans may use oleic acid or derivatives as a growth-promoting factor, perhaps during interactions with bacteria and amoebae in the soil or during colonization of the brain. It is particularly intriguing to speculate that oleic acid might contribute to the neurotropism of C. neoformans because it is the most abundant fatty acid in the brain and it functions in development, signaling, and dopamine production. It is also a component of the neurotransmitters N-oleoyl-dopamine and oleoylthanolamide (1, 21, 50, 58, 68, 77). In contrast, linoleic acid has complex roles in regulating C. neoformans growth. Intriguingly, this fatty acid induces apoptosis in mammalian tumor cell lines, and its inhibition of C. neoformans growth suggests that it may have a similar activity for the fungus (67). This possibility and the influence of oleic acid as a growth-promoting factor are fruitful areas for further investigation to determine the activities of fatty acids in fungal pathogenesis.

It is also possible that loss of β-oxidation altered the production of signaling lipids to influence host defense through immune modulation and interactions with phagocytic cells. A potential impact on signaling is intriguing because C. neoformans acquires fatty acids from macropHages and incorporates host arachidonic acid for prostaglandin (eicosanoid) production in a phospholipase B (PLB)-dependent manner (82). This process may dampen macrophage activation and enhance C. neoformans survival (52). C. neoformans does not have an identifiable cyclooxygenase for the production of oxypipins; however, the laccase enzyme for melanin synthesis is involved in the production of oxypipins and prostaglandins (14, 15, 66). As mentioned, cryptococcal PLB1 is responsible for the release of host arachidonic acid and is thought to interact with host cell membranes and lead to cell lysis (52, 82). Mutants with mutations in PLB1 are unable to colonize the lung, they show reduced survival during interactions with macrophages, and they are nonpathogenic. In addition to possible modulation of the host immune response, lipids are likely to have complex influences on C. neoformans. For example, it was recently found that phospholipids influence capsule size during cryptococcal interactions with macrophages and amoebae (8). Fatty acids or related molecules such as phospholipids and oxypipins are important signaling molecules in other fungi (3). For instance, fungal oxypipins (psl factors) are derived from oxidized oleic, linoleic, or linolenic acid and influence quorum sensing and the yeast-to-hypha transition in C. albicans, they regulate sexual development in Aspergillus species, and they show antibiotic activity in U. maydis (70, 74).

We have also examined β-oxidation in the plant pathogen U. maydis in parallel with our work on C. neoformans (see the accompanying article [33a]). U. maydis is also a basidiomycete fungus with both the peroxisomal and mitochondrial pathways. In both fungi, the mitochondrial pathway is important for the utilization of short-chain fatty acids and the peroxisomal pathway breaks down long-chain fatty acids, with the exception of the long-chain polyunsaturated fatty acid linoleic acid, which requires the activity of both pathways. Short- and medium-chain fatty acids are toxic at higher concentrations to both organisms, and defects in β-oxidation, whether in the mitochondrion or the peroxisome, lead to the accumulation of toxic intermediates. Furthermore, peroxisomal β-oxidation is important in both organisms for utilization of the alternative carbon source acetate, probably because of reduced acetyl-CoA production and/or disturbed transport of β-oxidation intermediates across the peroxisomal membrane. Finally, β-oxidation is glucose repressed, the β-oxidation genes are upregulated during infection, and the process is important for the virulence of both fungi.

Despite extensive similarities, C. neoformans and U. maydis have unique features that involve β-oxidation and contribute to their virulence. These differences likely reflect the unique ecological niches of the pathogens. That is, U. maydis is a biotrophic plant pathogen that may be well adapted to fatty acids as a carbon source because of interactions with the plant cuticular surface or developing maize seeds, which have a high lipid contents. In contrast, the primary ecological niche of C. neoformans is not well established, but it is known to associate with trees and pigeon guano, and it may have adapted to predation by soil amoebae (5, 7, 41, 51). This adaptation may also be important for interactions with macrophages in mammalian hosts, where the fungus may encounter lipids as signals or carbon sources. For U. maydis, fatty acids also act as signaling molecules that trigger the transition from yeast to filamentous growth that is important for plant infection, and peroxisomal β-oxidation contributes to this activity (32). In contrast, C. neoformans does not respond to fatty acids with filamentous growth, although oleic acid appears to be a growth factor. However, it uses fatty acid derivatives such as prostaglandins as signaling molecules for host-pathogen interactions. Additionally, β-oxidation influences secretion in C. neoformans as well as the major virulence factors melanin and the polysaccharide capsule. Peroxisomal β-oxidation is also important for cell wall integrity in C. neoformans but not in U. maydis. Despite the pleiotropic influence of β-oxidation in both fungi, our demonstration of the impact of the process on virulence suggests that fatty acid/lipid metabolism is a useful target for antifungal drug and fungicide development.

**ACKNOWLEDGMENTS**

We thank June Kwon-Chung for the qplΔ strains and Guanggan Hu and Melissa Caza for help with virulence assays. We are grateful to an anonymous reviewer for the suggestion of biotin as a rescue factor.

This work was supported by the Canadian Institutes of Health Research. J.W.K. is a Burroughs Wellcome Fund Scholar in Molecular Pathogenic Mycology.
REFERENCES

1. Alemany R, et al. 2010. Olive oils modulate fatty acid content and signaling protein expression in apolipoprotein E knockout mice brain. Lipids 45:53–61.

2. Bhambra GK, Wang ZY, Soanes DM, Wakley GE, Talbot NJ. 2006. Peroxiosomal carnitine acetyl transferase is required for elaboration of penetration hyphae during plant infection by Magnaporthe grisea. Mol. Microbiol. 61:46–60.

3. Brodhun F, Feussner I. 2011. Oxylipins in fungi. FEBS J. 278:1047–1063.

4. Byrnes EJ, III, Bartlett KH, Perfect JR, Heitman J. 2011. Cryptococcus gattii: an emerging fungal pathogen infecting humans and animals. Microbes Infect. 13:895–907.

5. Casadevall A. 2012. Amoeba provide insight into the origin of virulence in pathogenic fungi. Adv. Exp. Med. Biol. 710:1–10.

6. Chen LC, Blank ES, Casadevall A. 2005. Peroxisomal metabolic function is required for appressorium-mediated plant infection by Colletotrichum lagenarium. Plant Cell 13:1943–1957.

7. Chrisman CJ, Albuquerque P, Guimaraes AJ, Nieves E, Casadevall A. 2009. Phagosynthesis of Cryptococcus neoformans by, and nonlytic exocytosis from, Acanthamoeba castellanii. Appl. Environ. Microbiol. 76:6506–6506.

8. Chrisman CJ, Albuquerque P, Guimaraes AJ, Nieves E, Casadevall A. 2011. Phospholipids trigger Cryptococcus neoformans capsular enlargement during interactions with amoebae and macrophages. PLoS Pathog. 7:e1001247. doi:10.1371/journal.ppat.1001247.

9. Clouet P, Demizieux L, Gresti J, Degrace P. 2009. Comparative genome analysis across a kingdom of eukaryotic organisms: specialization and diversification in the fungi. Genome Res. 17:1809–1822.

10. Davidson NG, et al. 2002. A PCR-based strategy to generate integrative targeting alleles with large regions of homology. Microbiology 148:2607–2615.

11. Eigenheer RA, Jin Lee Y, Blumwald E, Phinney BS, Gelli A. 2007. Extracellular glycosylphosphatidylinositol-anchored mannoproteins and proteases of Cryptococcus neoformans, FEMS Yeast Res. 7:499–510.

12. Emerand AW, Voorn-Brouwer TM, Erdmann R, Knuaw WH, Tabak HF. 1991. Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl-CoA thiolase of Saccharomyces cerevisiae. Eur. J. Biochem. 200:113–122.

13. Erb-Downward JR, Huffnagle GB. 2007. Cryptococcus neoformans produces authentic prostaglandin E2 without a cyclooxygenase. Eukaryot Cell 6:346–350.

14. Erb-Downward JR, Noggle RM, Williamson PR, Huffnagle GB. 2008. The role of laccase in prostaglandin production by Cryptococcus neoformans. Mol. Microbiol. 68:1428–1437.

15. Fakas S, et al. 2011. Phosphatidate phosphatase activity plays a key role in protection against fatty acid-induced toxicity in yeast. J. Biol. Chem. 286:29074–29085.

16. Fan W, Kraus PR, Boffy MJ, Heitman J. 2005. Cryptococcus neoformans gene expression during murine macrophage infection. Eukaryot Cell 4:1420–1433.

17. Gollub EG, Trocha P, Liu PK, Sprinborn DB. 1974. Yeast mutants requiring oleate as only lipid supplement. Biochem. Biophys. Res. Com- mun. 56:471–477.

18. Gunkel K, van der Klei IJ, Barth G, Veenhuis M. 1999. Selective peroxisome degradation in Yarrowia lipolytica after a shift of cells from acetate/oleate/ethanolamine into glucose/ammonium sulfate-containing media. FEBS Lett. 451:1–4.

19. Hartig A, et al. 1992. Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of S. cerevisiae. Nucleic Acids Res. 20:5677–5686.

20. Heller A, et al. 2005. Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon. Neurosci. Lett. 376:35–39.

21. Hiltunen JK, Qin Y. 2000. Beta-oxygenation—strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim. Biophys. Acta 1484:117–129.

22. Houten SM, Wanders RJ. 2010. A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. J. Inherit. Metab. Dis. 33:469–477.

23. Hu G, Cheng PY, Sham A, Perfect JR, Kronstad JW. 2008. Metabolic adaptation in Cryptococcus neoformans during early murine pulmonary infection. Mol. Microbiol. 69:1456–1475.

24. Hu G, Kronstad JW. 2010. A putative P-type ATPase, Apr1, is involved in stress tolerance and virulence in Cryptococcus neoformans. Eukaryot. Cell 9:74–83.

25. Hynes MJ, Murray SL, Kheu GS, Davis MA. 2008. Genetic analysis of the role of peroxisomes in the utilization of acetate and fatty acids in Aspergillus nidulans. Genetics 178:1355–1369.

26. Idnurm A, Giles SS, Perfect JR, Heitman J. 2007. Peroxisome function regulates growth on glucose in the basidiomycete fungus Cryptococcus neoformans. Eukaryot. Cell 6:650–72.

27. Janbon G. 2004. Cryptococcus neoformans capsule biosynthesis and regulation. FEMS Yeast Res. 4:765–771.

28. Karst F, Lacroute F. 1977. Ertosterol biosynthesis in Saccharomyces cerevisiae mutants deficient in the early steps of the pathway. Mol. Gen. Genet. 154:269–277.

29. Kidd SE, et al. 2007. Characterization of environmental sources of Cryptococcus gattii in British Columbia, Canada, and the Pacific Northwest. Appl. Environ. Microbiol. 73:1433–1443.

30. Kimura A, Takano Y, Furusawa I, Okuno T. 2001. Peroxisomal metabolic function is required for appressorium-mediated plant infection by Colletotrichum lagenarium. Plant Cell 13:1943–1957.

31. Klose J, Kronstad JW. 2006. The multifunctional beta-oxidation enzyme is required for full symptom development by the biotrophic maize pathogen Ustilago maydis. Eukaryot. Cell 5:2047–2061.

32. Magliano P, Flipphi M, Arpat BA, Delessert S, Poirier Y. 2006. Peroxisome function is required for appressorium-mediated plant infection by Colletotrichum lagenarium. Plant Cell 13:1943–1957.

33. Kretschmer M, Klose J, Kronstad JW. 2012. Defects in mitochondrial and peroxisomal β-oxidation influence virulence in the maize pathogen Ustilago maydis. Eukaryot Cell 11:1055–1066.

34. Kronstad JW, et al. 2011. Expanding fungal pathogenesis: Cryptococcus breaks out of the opportunistic box. Nat. Rev. Microbiol. 9:193–203.

35. Kozubowski I, Heitman J. 2012. Profiling a killer, the development of Cryptococcus neoformans, FEMS Microbiol. Rev. 36:78–94. doi:10.1111/ j.1574-6976.2011.00286.x.

36. Legras JL, et al. 2004. The multifunctional beta-oxidation enzyme is required for full symptom development by the biotrophic maize pathogen Ustilago maydis. Eukaryot. Cell 3:570–574.

37. Litvintseva AP, et al. 2011. Evidence that the human pathogenic fungus Cryptococcus neoformans var. grubii may have evolved in Africa. PLoS One 6:e19688. doi:10.1371/journal.pone.0019688.

38. Lockshon D, Surface LE, Kerr EO, Kaebelme L, Kennedy BK. 2007. The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics 175:77–91.

39. Lorenz MC, Fink GR. 2001. The glyoxylate cycle is required for fungal virulence. Nature 412:83–86.

40. Legras JL, et al. 2004. The multifunctional beta-oxidation enzyme is required for full symptom development by the biotrophic maize pathogen Ustilago maydis. Eukaryot. Cell 3:570–574.

41. Mannaerts GP, Van Veldhoven PP, Casteels M. 2006. Peroxisome function is required for full symptom development by the biotrophic maize pathogen Ustilago maydis. Eukaryot. Cell 3:570–574.

42. Manders JP, Flibphi M, Arpat BA, Delessert S, Poirier Y. 2011. Contributions of the peroxisome and β-oxidation cycle to biont synthesis in fungi. J. Biol. Chem. 286:42133–42140.

43. Margolles S, Vandevelde H, Pester K, Poirier Y. 2011. Peroxisomal lipid degradation via beta- and alpha-oxidation in mammals. Cell Biochem. Biophys. 32:73–87.

44. Masi LN, Portioli-Sanches EP, Lima-Salgado TM, Curi R. 2011. Toxicity of fatty acids on ECV-304 endothelial cells. Toxicol. In Vitro 25:2140–2146.

45. Morel F, Laruquin G, Lunardi J, Dusszynski J, Vignais PV. 1974. An
appraisal of the functional significance of the inhibitory effect of long chain acyl-CoAs on mitochondrial transports. FEBS Lett. 39:133–138.

50. Navarrete CM, et al. 2010. Endogenous N-acyl-dopamines induce COX-2 expression in brain endothelial cells by stabilizing mRNA through a p38 dependent pathway. Biochem. Pharmacol. 79:1805–1814.

51. Nielsen K, De Obaldia AL, Heitman J. 2007. Cryptococcus neoformans mates on pigeon guano: implications for the realized ecological niche and globalization. Eukaryot. Cell 6:949–959.

54. Okabayashi K, Kano R, Watanabe S, Hasegawa A. 2006. The carnitine acyltransferases: modulators of acyl-CoA-dependent reactions. Biochem. Soc. Trans.

61. Ramsay RR. 2000. The carnitine acyltransferases: modulators of acyl-CoA-dependent reactions. Biochem. Soc. Trans. 28:182–186.

62. Reiser K, Davis MA, Hynes MJ. 2007. Peroxisomes, lipids and lipid metabolism and lipotoxicity. Biochim. Biophys. Acta 1801:272–280.