Peroxisome Function Regulates Growth on Glucose in the Basidiomycete Fungus Cryptococcus neoformans\(^\text{\textdagger}\)

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The function of the peroxisomes was examined in the pathogenic basidiomycete Cryptococcus neoformans. Recent studies reveal the glyoxylate pathway is required for virulence of diverse microbial pathogens of plants and animals. One exception is C. neoformans, in which isocitrate lyase (encoded by ICL1) was previously shown not to be required for virulence, and here this was extended to exclude also a role for malate synthase (encoded by MLS1). The role of peroxisomes, in which the glyoxylate pathway enzymes are localized in many organisms, was examined by mutation of two genes (PEX1 and PEX6) encoding AAA (4TPases associated with various cellular activities)-type proteins required for peroxisome formation. The pex1 and pex6 deletion mutants were unable to localize the fluorescent DsRED-SKL protein to peroxisomal punctate structures, in contrast to wild-type cells. pex1 and pex6 single mutants and a pex1 pex6 double mutant exhibit identical phenotypes, including abolished growth on fatty acids but no growth difference on acetate. Because both icl1 and mlb1 mutants are unable to grow on acetate as the sole carbon source, these findings demonstrate that the glyoxylate pathway can function efficiently outside the peroxisome in C. neoformans. The pex1 mutant exhibits wild-type virulence in a murine inhalation model and in an insect host, demonstrating that peroxisomes are not required for virulence under these conditions. An unusual phenotype of the pex1 and pex6 mutants was that they grew poorly with glucose as the carbon source, but nearly wild type with galactose, which suggested impaired hexokinase function and that C. neoformans peroxisomes might function analogously to the glycosomes of the trypanosomid parasites. Deletion of the hexokinase HXK2 gene reduced growth in the presence of glucose and suppressed the growth defect of the pex1 mutant on glucose. The hexokinase 2 protein of C. neoformans contains a predicted peroxisome targeting signal (type 2) motif; however, Hxk2 fused to fluorescent proteins was not localized to peroxisomes. Thus, we hypothesize that glucose or glycolytic metabolites are utilized in the peroxisome by an as yet unidentified enzyme or regulate a pathway required by the fungus in the absence of peroxisomes.

The paucity of applicable antimicrobial agents and the growing resistance to existing agents raise concern about the long-term control of infectious diseases, particularly those caused by fungi. The search for novel genes that lack human counterparts and are essential for pathogen survival or virulence and the design of molecules that target these genes or encoded proteins represent an approach to new drug discovery. An exciting discovery towards this goal was the identification of the glyoxylate pathway as playing a role in microbial virulence (reviewed in reference 43). This pathway catalyzes the conversion of isocitrate to malate and succinate and is essential for the utilization of two-carbon molecules (e.g., ethanol and acetate) as carbon sources. The first enzyme in the pathway, isocitrate lyase, is required for virulence of Mycobacterium tuberculosis and Candida albicans in mice (44, 47, 50), as well as for the fungi Colletotrichum lagenarium, Leptosphaeria maculans, and Magnaporthe grisea in plants (2, 27, 78). The second enzyme in the pathway, malate synthase, is involved in virulence in the bacterium Rhodococcus fascians and the fungus Stagonospora nodorum towards plants (67, 75). The pathway is absent in vertebrates; hence, drugs targeting it, perhaps aided by the crystal structure (5, 65, 66) or gene-specific inactivation (40), may therefore be broad-spectrum and safe human therapeutics. Although plants contain glyoxylate pathway enzymes, there is also potential for application in agricultural settings, given the temporal expression of the pathway enzymes in plants (e.g., during seed germination and plant senescence).

Peroxisomes are single-membrane-bound organelles associated with a suite of cellular functions, including peroxide detoxification, β-oxidation of fatty acids, and utilization of D-amino acids (reviewed in reference 46). The glyoxylate pathway in plants and fungi is most often a component of peroxisome function (53, 81). Peroxisomes are also essential for human health. Twelve complementation groups have been identified in humans with deficiencies in peroxisome function, most of which are lethal or lead to early death or debilitating disease (79). In otherwise healthy individuals, peroxisomes are required for metabolism of fatty acids and produce reactive oxygen as part of this process. Many aspects of the functions of the human proteins required for peroxisome formation have been elucidated by using fungi, particularly Saccharomyces cerevisiae, as model systems for peroxisome function, including the identification of the human genes mutated in 9 of the 12 complementation groups (reviewed in reference 22).

Peroxisomes can be required for microbial virulence. Deletion of pex6 in the fungus Colletotrichum lagenarium reduces

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disease on bean leaves by impairing appressorium function during the initial stages of infection (36). Recently, a PEx6 homolog and a peroxisome-targeted acetyltransferase have been shown to be required for appressorium formation and pathogenicity of the major rice pathogen *Mogonaportha grisea* (4, 60). Similarly, hex1 mutations impair appressorium formation in *M. grisea* (68). Hext is a protein required for formation of Woronin bodies, which are specialized peroxisomes found only in filamentous ascomycete fungi, which function to block septal pores in damaged hyphae. The KinetoPlastida parasites, such as those in the *Trypanosoma* and *Leishmania* genera, contain an unusual type of peroxisome named the glycosome because up to seven of the nine enzymes for glycolysis are targeted within them, and no Kreb's cycle occurs within these organisms. Reduced expression of genes required for peroxisome assembly is lethal to trypanosomes as a consequence of enzyme mislocalization to the cytoplasm, and as such, the glycosomes are proposed drug targets for these parasites (15, 23, 49).

One concern with developing a drug that targets the glyoxylate pathway enzymes was the discovery that *Cryptococcus neoformans* does not require isocitrate lyase for virulence, despite the observation that the transcript is highly up-regulated in the presence of glucose. Does not require isocitrate lyase for virulence, despite the observation that the transcript is highly up-regulated in the presence of glucose.

**Materials and Methods**

**Fungal strains.** *Cryptococcus neoformans* serotype A strains H99 (MAT*α*), KN99a, KN99a, KN3B7#12 (MATα), the eight backcross to generate serotype *A* congenic parents KN99a and KN99a (52), and *icl1* (MATα) mutant (61) were used. Serotype D strains JEC20 (MATα) and JEC21 (MATα) were used to assay the mating type of serotype A strains (39). *Saccharomyces cerevisiae* BY4743 wild-type reference and *icl1α* and *peX1α* mutant diploid strains were obtained from the *S. cerevisiae* gene deletion set (18). The *C. neoformans* strains used in this study are listed in Table 1.

**Database analyses of the *C. neoformans* genome.** The protein sequences encoded by the *S. cerevisiae* *MLS1*, *PEX1*, *PEX5*, *PEX6*, and *PEX7* genes were used to search the *C. neoformans* genome database (www.tigr.com) (42) with BLASTp and tBLASTn using default parameters. The sequences of the nine enzymes required for glycolysis in *S. cerevisiae* were likewise used in searches against the *C. neoformans* genome databases. The subcellular localizations of the putative *C. neoformans* homologs were predicted using PSORT II software and manual searches of peroxisomal targeting signals.

**Creation of *C. neoformans* mutant strains.** Mutations of the *HXK2*, *MLS1*, *PEX1*, *PEX5*, *PEX6*, and *PEX7* genes were isolated by replacing 95 to 100% of the coding regions of these genes with a cassette conferring resistance to neomycin (pPZP-NEO1) (76) via biolistic transformation. The mutation was confirmed by PCR and Southern blot analysis with DNA extracted using cetyltrimethylammonium bromide buffer (58). A *peX1* mutant was crossed to the congenic isolate KN3B7#12, and basidiospores from the cross were isolated by micromanipulation and analyzed to obtain a *peX1* MAT*α* strain. Crosses were conducted on V8 medium (5% Campbell's V8 juice, 0.5 g/liter KH₂PO₄, 40 g/liter Bacto-agar, pH 5.5) for up to 3 weeks in the dark. Mating was unaffected in *peX1* mutants, including *peX1* × *peX5* crosses. The *peX1* MAT*α* strain was crossed to a *peX6* strain, and the progeny of this cross were analyzed by Southern blotting to identify a double *peX1* *peX6* mutant strain. An *mls1*::NAT disruption allele was created by overlap PCR and subcloning. The cassette was cloned into plasmid pPPP-201Bk and introduced into cells of *Agrobacterium tumefaciens* strain LBA4404 or EHA105 by electroporation. *Agrobacterium*-mediated transformation of *C. neoformans* was performed as described previously (28). Attempts to mutate *MLS1* using *Agrobacterium*-mediated transformation were unsuccessful. The *mls1*::NAT construct was therefore amplified by PCR using the plasmid as template and used successfully to delete the *MLS1* gene via biolistic transformation. The *peX1* mutation was complemented by the ectopic introduction of a wild-type copy of the *PEX1* gene fused to a cassette conferring resistance to neomycin via biolistic transformation (14). The *icl1* mutation was complemented by the ectopic introduction of a wild-type copy of the *HXK2* gene fused to the cassette conferring resistance to neomycin (pPPZ-NEO1) (76) via *Agrobacterium*-mediated transformation.

**Fluorescent protein constructs and microscopy.** To assess organelle localization, overlap fusion products were created for green or red fluorescent proteins expressed from the *C. neoformans* histone H3 promoter using primers listed in Table 3. Green fluorescent protein (GFP) (S65T) was provided by Tian Lian and Nichols as a derivative of pDsRED-Express (BD Biosciences, Palo Alto, CA). A peroxisome-targeted fluorescent protein (DsRED-SKL) driven by the histone H3 promoter and terminator was fused to the neomycin resistance marker using overlap PCR and introduced into *C. neoformans* strain KN99a by biolistic transformation. The same strategy was used to create a DsRED construct without the -SKL-terminal amino acids. Overlap fusion products to express GFP or DsRED were subcloned into the Sac site of pPZP-NATce (76), as illustrated in Fig. 1A. To visualize the cell wall, strains were grown in YPD (yeast extract, peptone, dextrose) medium with Calcofluor white (40 μg/ml) for 5 min and washed three times in phosphate-buffered saline. Cells were mounted in Vectashield (Vector Laboratories, California). DsRED or GFP protein and Calcofluor fluorescence was examined using a Zeiss Axioskop 2 Plus fluorescence microscope and pho-

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**Table 1. *C. neoformans* strains used in this study**

| Name                  | Genotype           | Background/parents |
|-----------------------|--------------------|-------------------|
| AI24                  | pex1::NAT MATα     | H99               |
| AI26                  | pex1::NAT MATα     | AI24 × KN3B7#12   |
| AI28                  | pex6::NAT MATα     | H99               |
| AI31                  | pex1::NAT pex6::NAT MATα | AI26 × AI28     |
| AI47                  | pex1::NAT PEX1-NAT MATα | AI24             |
| AI57                  | mls1::NAT MATα     | KN99a             |
| AI100                 | P15::DsRED-NEO MATα | KN99a             |
| AI100                 | P15::DsRED-SKL-NEO MATα | KN99a          |
| AI104                 | P15::DsRED-SKL-NEO MATα | AI26 × AI100    |
| AI105                 | pex6::NAT P15::DsRED-SKL-NEO MATα | AI28 × AI104 |
| AI107                 | pex5::NAT P15::DsRED-SKL-NEO MATα | AI61 × AI104 |
| AI110                 | hks2::NAT MATα     | KN99a             |
| AI110                 | hks2::NAT pex1::NAT MATα | AI26 × AI110   |
| AI118                 | hks2::NAT HXK2-NAT MATα | AI110           |
| AI120                 | hks2::NAT HXK2-NAT pex1::NAT MATα | AI26 × AI118 |
| AI125                 | pex7::NAT MATα     | KN99a             |
| AI127                 | pex7::NAT MATα     | AI125 × KN99a    |
| AI141                 | pex7::NAT P15::DsRED-SKL-NEO MATα | AI104 × AI125 |
| AI142                 | P15::GFP-NAT MATα  | KN99a             |
| AI143                 | P15::DsRED-NAT MATα | KN99a             |
| AI144                 | P15::HXK2-GFP-NAT MATα | KN99a          |
| AI145                 | P15::HXK2-DsRED-NAT MATα | KN99a          |
The NAT cassette was ligated into the KpnI-XbaI sites (boldface).

For overlap PCR, primers 1 and 2 were used on JEC21 genomic DNA, primers 3 and 4 were used on NEO plasmid, and primers 5 and 6 were used on the DsRED or GFP plasmid. Overlap PCR was performed with primers 1 and 6.

Strong expression was observed in the integrated transformants, as judged by fluorescence microscopy and flow cytometry (Fig. 1B and C). The DsRED fluorescence varied among transformants with the same construct, indicating that the site of integration on the genome affects expression levels (Table 1).

**TABLE 2. Oligonucleotide primers for overlap PCR to make**

| Primer name | Primer sequence (5′→3′)* |
|-------------|--------------------------|
| **PEX1** (CND06230) | |
| disruption | |
| 1. JOHE8689 | CACAGTTCTGTCACCTAGTTCG |
| 2. JOHE8724 | AGCTCACTATCTCAGCAGACCCAGAT |
| 3. JOHE8719 | GCCAAGTCGATCTGTGGTGCG |
| 4. JOHE8722 | GTAAGTGACCTGGCAGAAGAG |
| 5. JOHE8721 | TAGTTTCATCCTCCTCGCCGAAG |
| 6. JOHE8690 | GCAAGAAGACCTGAAAGG |

| **PEX6** (CNI03110) | |
| disruption | |
| 1. JOHE8691 | GCTTCTCAATGTGATCG |
| 2. JOHE8723 | CGTCTAGGCAAGGCTG |
| 3. JOHE8726 | GAGTTTGACC |
| 4. JOHE8725 | TAGTTTCATCCTCCTCGCCGAAG |
| 5. JOHE8692 | ACTTTGCCGAGGAAAG |

| **MSL1** (CNBL2980) | |
| disruption | |
| 1. JOHE10129 | GTCGACATTTGAACAGTATC |
| 2. JOHE10132 | CACAAACGA |
| 3. JOHE11465 | GAAGAAGCATGTGGTCAGG |
| 4. JOHE11516 | GTCCTCGGAGGAGGCCATGG |
| 5. JOHE11517 | GTCCTCGGAGGAGGCCATTG |
| 6. JOHE10130 | GTGACGACAAAACAGATAC |

| **PEX5** (CNI0870) | |
| disruption | |
| 1. JOHE11465 | GAAAAGACCATGTGTCAGG |
| 2. JOHE11466 | GTCTATGCTGTTTCTCGACTGAGT |
| 3. M13R | CAGGAAACAGCTATGAC |
| 4. M13F | GATACAATAA |
| 5. JOHE11467 | CTGGCCGTCGTTTCCGGCTTAAAG |
| 6. JOHE11469 | AAGCAGGAGGCCGTTGAC |

| **PEX7** (CNA04980) | |
| disruption | |
| 1. JOHE12196 | TGGCGCGGTTCGTTAACAGC |
| 2. JOHE12197 | CTGCGCGGTTCGTTTACAGG |
| 3. M13R | CAGGAAACAGCTATGAC |
| 4. JOHE11465 | GAAAAGACCATGTGTCAGG |
| 5. JOHE11466 | GTCTATGCTGTTTCTCGACTGAGT |
| 6. JOHE11469 | AAGCAGGAGGCCGTTGAC |

| **HXX2** (CNBB3020) | |
| disruption | |
| 1. JOHE13893 | TTTAGACTTCAGTGGGAAG |
| 2. JOHE13894 | GGGTACTTGTGCTTGCTCCCAGTCTG |
| 3. JOHE11866 | GGGAGGACCTCAGACAG |
| 4. JOHE86777 | GAAGAAGATGTGAAAGGAG |
| 5. JOHE13895 | CCTGTCTTCTACATCTCTCTGCT |
| 6. JOHE13896 | TTTGATGTTTAGTGGC |

* Primers 1 and 2 and 5 and 6 were used on C. neoformans H99 genomic DNA, and primers 3 and 4 were used on a plasmid conferring resistance to nourseothricin. Equimolar amounts of the three products were mixed, and the overlap PCR was performed with primers 1 and 6.

* The NAT cassette was ligated into the Km!-Xbal sites (boldface).

* Includes signature tag JKL 211.

For overlap PCR, primers 1 and 2 were used on JEC21 genomic DNA, primers 3 and 4 were used on NEO plasmid, and primers 5 and 6 were used for the full overlap PCR. For overlap constructs that were subcloned using Sacl sites into the Agrobacterium T-DNA vector, primers 1 and 2 and 5 and 6 were used on JEC21 genomic DNA and primers 3 and 4 were used on the Neo or GFP plasmid. Overlap PCR was performed with primers 1 and 6.

* SacI sites are in boldface.

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***Contribution of P H3-DsRED***

**TABLE 3. Oligonucleotide primers for overlap PCR to make**

| Primer name | Primer sequence (5′→3′)* |
|-------------|--------------------------|
| **Constitution of P H3-DsRED** | |
| 1. JOHE12993 | ATGCCCTTGACTGCTTAAG |
| 2. JOHE13185 | CACACCTGTCTGCTGATT |
| 3. JOHE13184 | CACACCTGTCTGCTGATT |
| 4. JOHE13183 | CACACCTGTCTGCTGATT |
| 5. JOHE13182 | CACACCTGTCTGCTGATT |
| 6. JOHE13181 | CACACCTGTCTGCTGATT |

* For DsRED-NEO overlap PCR, primers 1 and 2 were used on the DsRED plasmid, primers 3 and 4 were used on the NEO plasmid, and primers 5 and 6 were used on JEC21 genomic DNA. Primers 4 and 5 were used for the full overlap PCR. For overlap constructs that were subcloned using Sacl sites into the Agrobacterium T-DNA vector, primers 1 and 2 and 5 and 6 were used on JEC21 genomic DNA and primers 3 and 4 were used on the DsRED or GFP plasmid. Overlap PCR was performed with primers 1 and 6.

* Sacl sites are in boldface.

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tigraphyed with an AxioCam MRM digital camera. For fluorescence, the Zeiss filters for 4.6-6-diamino-2-phenylindole (DAPI) (Calcofluor white staining: excitation λmax, 365 nm), GFP gr (excitation, 450 to 480 nm), and both tetramethyl rhodamine isothiocyanate (TRITC) (DsRED; excitation 510 to 560 nm) and Texas red (DsRED; excitation, 530 to 580 nm) were used. As an independent measure of fluorescence, strains grown overnight in liquid YPD were sorted for size and fluorescence by flow cytometry.

In C. neoforms, GFP and DsRED have been used previously to study protein localization or expression (10, 80). However, use of GFP has been problematic and few published vectors are available for expression in C. neoformans. The constructs were introduced in A. tumefaciens strain EHA105, and these strains were used to transform C. neoforms. Weak autofluorescence was observed in wild-type cells, particularly associated with the plasma membrane. While cytoplasmic red fluorescence was observed with the DsRED construct, green fluorescence was very weak with the GFP construct, despite the same promoter being used to drive expression (Fig. 1B). The reasons are unknown; however, fusion of the C. neoforms Hx2 protein to the N terminus dramatically enhanced levels of fluorescence (Fig. 1B and 8C), suggesting a requirement for C. neoforms nucleotide bias or introns for stable expression. A similar result has been observed for GFP expression in other basidiomycete fungi,
including *Agaricus bisporus* and *Coprinus cinereus*, in which inclusion of introns is hypothesized to stabilize protein expression (6). Recently, the GFP gene has been optimized for *C. neoformans* codon usage to enhance fluorescence (41).

**In vitro phenotypic analysis.** The growth of strains was compared on YPD (yeast extract, peptone, dextrose) and YNB (yeast nitrogen base) agar medium supplemented with different carbon sources: glucose (0.2%, 0.5%, 1%, or 2%), fructose (2%), mannose (2%), galactose (2%), sodium acetate (1% or 2%), and oleic acid (5 mM plus 1% tergitol). Melanin and capsule were assayed on L-DOPA medium (100 mg/liter) and in low-iron medium (with the chelator EDDHA). Growth curves were conducted on cells that were grown overnight in liquid YPD medium, washed, and added to yeast nitrogen base with different carbon sources in shaking or rotating cultures at 30°C or 37°C, using starting inocula at an optical density at 600 nm (OD$_{600}$) of 0.1.

**Virulence assays.** For murine assays, *C. neoformans* cells were used to infect 4- to 6-week-old female A/Jcr mice (NCI/Charles River Laboratories) by nasal inhalation. Ten animals each were inoculated with a 50-$\mu$L inoculum containing $1 \times 10^5$ yeast cells of KN99a, H99, mls1, or pex1 PEX1 complemented strains. Animals were examined daily and sacrificed when signs of morbidity were observed. The experiment was double blinded, such that the strain genotypes remained anonymous during inoculum preparation and administration into animals, as well as until all animals were sacrificed. The murine experimental protocol was approved by the Duke University Animal Use Committee.

The wax moth virulence assay followed the previous protocol (51), with *Galleria mellonella* larvae purchased from Vanderhorst, Inc. (St. Marys, Ohio). The inoculum was $1 \times 10^5$ yeast cells, and larvae were incubated at 37°C postinoculation. Larvae were examined daily, and those not responding to touch were scored as inviable.

**RESULTS**

A malate synthase mutant is virulent, demonstrating the glyoxylate pathway is not required for virulence. The glyoxylate pathway enzyme isocitrate lyase is essential for virulence in diverse pathogenic organisms. One exception is *C. neoformans*: despite high transcription of *ICL1* in the host, no reduction in virulence was observed when the gene was mutated (61). Here a mutation was isolated in the gene encoding the second enzyme unique to this pathway, malate synthase (*MLS1*), to test if this gene played a role in virulence. We hypothesized that the high transcription of *ICL1* in vivo in an *mls1* mutant background may cause metabolic perturbation to the fungus and that *icl1* and *mls1* mutations might confer different phenotypes. Like the *icl1* mutant, the *mls1* mutant was unable to grow on acetate as the sole carbon source (Fig. 2A). A wild-type strain and an *mls1* mutant strain were inoculated into mice or an alternative insect host (wax moth larvae), and survival was monitored daily. Mice or wax moths infected with either strain succumbed to lethal infection with the same efficiency as the wild type, indicating that *MLS1* plays no role in virulence under these conditions (Fig. 2B and C). Thus, the two components of the glyoxylate pathway, isocitrate lyase and malate synthase, have similar functions in *C. neoformans* to promote growth on the two-carbon substrate acetate, but appear to play no role in virulence in a murine inhalation or insect assay.

Protein localization to peroxisomes is impaired by mutating the *PEX1* or *PEX6* genes. The glyoxylate pathway is generally considered to be localized within the peroxisomes of plants and fungi (53, 81). We therefore aimed to disrupt peroxisomes to...
test their function with respect to the glyoxylate pathway and virulence. The completed genome sequence of \textit{C. neoformans} (35) was searched for homologs of highly conserved peroxin (\textit{PEX}) genes that have been identified from organisms as required for peroxisome formation or function. To test the function of peroxisomes in the biology of \textit{C. neoformans}, two key genes required for peroxisome function in other organisms were disrupted. \textit{Pex1} and \textit{Pex6} homologs are functionally-related AAA (\textit{A}TPases \textit{a}ssociated with various cellular \textit{a}ctivities)-type proteins with a role in the assembly of small peroxisomal vesicles into peroxisomes, aiding vesicle fusion in an ATP-dependent manner, and/or protein import into the peroxisomes (32, 59, 64, 71). The \textit{PEX1} and \textit{PEX6} genes of \textit{C. neoformans} were mutated by targeted disruption, and a \textit{pex1 pex6} double mutant was obtained as a meiotic segregant from a \textit{pex1} \times \textit{pex6} cross. The \textit{pex1} mutation was complemented by reintroduction of a wild-type copy of the \textit{PEX1} gene.

Peroxisome function was assayed based on localization of a fluorescent protein into these organelles. Addition of a peroxisome targeting signal 1 (PTS1), such as variants of the tripeptide -SKL, to the C-terminal end of proteins can enable their translocation into the peroxisome (16). Even conjugation of the -SKL tripeptide to gold beads targets their import into peroxisomes (77). \textit{C. neoformans} \textit{Icl1} terminates with -HKL. When a DsRed-SKL protein was expressed in a wild-type background, a punctate fluorescence pattern was observed, indicating that the DsRED protein was localized to peroxisomes (Fig. 3). Punctate fluorescence was observed in cells growing on normal carbon sources (i.e., glucose) and did not require induction by fatty acid carbon sources, which is consistent with peroxisomes being present in the related \textit{Cryptococcus humicolus} species under glucose conditions (30) but in contrast to several other fungi in which peroxisomes are either not formed or are degraded in the presence of glucose (1, 24, 73).

The DsRED-SKL strain with punctate peroxisomal fluorescence was crossed to the \textit{pex1} mutant, and meiotic progeny (a total of 18) were isolated. Half of those that were fluorescent (4/9) showed a pattern of discrete localization, whereas the other half (5/9) showed fluorescence throughout the cell. Those five progeny with diffuse cytoplasmic fluorescence all bore a disruption of the \textit{pex1} gene. In a similar genetic approach (38 progeny analyzed: 12/22 localized, wild type for \textit{PEX6} wild type; 10/22 nonlocalized, \textit{pex6} deletion), no distinct localization was observed when the DsRED-SKL protein was
expressed in the pex6 background (Fig. 3). Thus, pex1 or pex6 deletions impair the transport of a peroxisomal marker protein and likely impact the function of peroxisomes in C. neoformans.

**PEX1 and PEX6 are required for growth on minimal medium containing glucose, but not acetate.** The phenotypes of the pex1 and pex6 mutant strains were compared to those of the wild-type strain (H99), the pex1 PEX1 complemented strain, and the icl1 and mls1 mutants (Fig. 4; Table 4). As noted above, the mls1 and icl1 strains were unable to grow on acetate as their sole carbon source. The pex1, pex6, and pex1 pex6 mutant strains of C. neoformans showed growth equivalent to that of the wild type on YNB with acetate. Wild-type C. neoformans exhibits slow growth on many fatty acids (19); the icl1, mls1, and pex mutants all show a marked reduction in growth on YNB with the fatty acid oleic acid as the carbon source (Table 4). Curiously, pex mutant strains all grew poorly on YNB media with glucose as the sole carbon source. Growth of pex1 or pex6 mutant strains was also inhibited on medium containing a mixture of glucose and acetate, which can be interpreted as the presence of glucose either is toxic to the cells or causes carbon catabolite repression of genes required for acetate utilization (Table 4). To ensure that the phenotype of the pex1 and pex6 mutants in the presence of glucose was not due to an artifact of medium preparation, growth of the C. neoformans strains was compared to that of S. cerevisiae icl1 and pex1 mutant strains. The S. cerevisiae pex1 mutant showed equal growth to the reference strain on YNB with either glucose or acetate, while the icl1 mutant was unable to grow on acetate (Fig. 4).

**Pex5 functions in -SKL targeting to peroxisomes, while Pex7 functions in the growth defect seen in pex1 or pex6 mutants.** To elucidate which proteins are responsible for the glucose-defective growth phenotype of pex1 and pex6 mutants, two genes that control targeting of proteins to peroxisomes were mutated: PEX5 and PEX7, which were identified based on their similarity to the S. cerevisiae homologs. Pex5 is a conserved protein that binds to the peroxisome-targeting signal 1 (PTS1) located at the C-terminal end of proteins and assists in their import into the peroxisome. There are two putative homologs of PEX5 in C. neoformans, similar to the situation in humans, while there is only one copy in ascomycete fungi (34). There is a single copy of PEX7, which encodes the protein that binds a second class of peroxisome targeting signal (PTS2). The gene encoding the DsRed-SKL protein was crossed into the pex5 and pex7 mutant backgrounds. Localization was severely impaired in pex5 cells. However, occasional punctate localization was observed, which may be consistent with a partially functional second homolog of PEX5 in the C. neoformans genome (Fig. 5B). Punctate localization equivalent to that seen in DsRED-SKL in wild-type cells was observed in the pex7 mutant background, indicating that Pex7 is dispensable for targeting this type of protein to the peroxisomes.

Growth rates of the wild-type and pex1, pex5, and pex7 mutant strains were compared on minimal media supplemented with glucose or acetate (Fig. 5A) and other carbon sources (Table 4). The pex5 mutant grew like the wild type, while the pex7 mutant exhibited reduced growth on YNB with glucose, although not as dramatically as that seen with the pex1 mutant (Fig. 5A). To further illustrate this reduction in growth rate,

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**TABLE 4. Qualitative growth rates of C. neoformans strains grown on yeast nitrogen base agar supplemented with 2% glucose, acetate, or galactose, or 1% of each in mixed carbon media**

| Strain type          | Glucose | Acetate | Galactose | Acetate + glucose | Acetate + galactose | Glucose + galactose | Oleic acid |
|----------------------|---------|---------|-----------|------------------|--------------------|---------------------|------------|
| Wild type (KN99a/H99) | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +          |
| icl1                 | ++++    | –       | ++++      | ++++             | ++++               | ++++                | +/–        |
| mls1                 | ++++    | –       | ++++      | ++++             | ++++               | ++++                | +/–        |
| pex1                 | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | –/–        |
| pex5                 | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +/–        |
| pex6                 | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | –/–        |
| pex7                 | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | –/–        |
| pex1 pex6            | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +/–        |
| hxk2                 | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +/–        |
| hxs2 hxk2            | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +/–        |
| pex1 hxk2            | ++++    | ++++    | ++++      | ++++             | ++++               | ++++                | +/–        |

* Oleic acid was added at 5 mM with 0.5% tergitol. +/– indicates very poor growth. Growth was observed at 24, 48, and 72 h at 30°C and 37°C.
strains were assessed in YNB media with glucose or galactose as the carbon sources. The pex7 mutant exhibits a modest reduced growth rate over the first 24 h of culture (Fig. 5C). The pex5 mutant showed an equivalent reduction in growth on oleic acid, as seen in strains bearing pex1 or pex6 deletions. In contrast, deletion of pex7 had no effect on growth on oleic acid. These data suggest that Pex5 mediates the targeting of the PTS1 proteins to the peroxisomes, including those for fatty acid utilization. Second, the data suggest that the PTS2 (Pex7-dependent) pathway of protein import into the peroxisomes could in part mediate the reduced growth rates seen in pex1 or pex6 mutant backgrounds on glucose medium.

**Peroxisome function is not required for C. neoformans virulence in two animal host models.** The pex1 or pex6 mutants show a growth defect on YNB medium, which is the standard minimal medium used for this fungus. We hypothesized that this defect might compromise the virulence of these strains. The *C. neoformans* mutants were examined for in vitro changes in traits most commonly associated with virulence. Growth at mammalian temperature (37°C) was equal to that at 30°C. Melanin and capsule were produced normally in the *C. neoformans* mutants. The pex1 mutant strain was tested for virulence in the murine nasal inhalation model. Surprisingly, wild-type, pex1 mutant, and pex1 PEX1 complemented strains showed equivalent virulence in this assay (Fig. 6A). As a second test of virulence, the wax moth larvae model was used. As with the murine inhalation model, no decrease in virulence was observed in the pex1 mutant background (Fig. 6B). Thus, in two diverse models there is no evidence for a role of peroxisomes in *C. neoformans* virulence.

**C. neoformans peroxisomes are required for efficient growth on glucose and other monosaccharides.** The most unexpected and curious phenotype of the *C. neoformans* pex1 or pex6 mutants was represented by their growth defects on minimal medium (YNB). Peroxisomes have numerous and diverse functions in different organisms (46). Disruption of peroxisome function does not usually impair fungal growth under rich growth conditions (25, 32, 36): these data on *C. neoformans* provide a rare exception. A second exception is the ascomycete fungus *Penicillium chrysogenum*. In this fungus, a strain with a mutation in the *PEX5* gene, required for transport of peroxisomally targeted proteins, shows poor growth, while isolation of mutants with mutation in *PEX1* or *PEX6* was not possible, suggesting that these genes may be essential (31, 33). A recent report from another ascomycete, *M. grisea*, suggests that growth rate of the pex6 mutant strain is also slightly reduced (60).

There are two possible explanations for the adverse effects of glucose on growth of the pex1 and pex6 mutants. The first is a defect in response to oxidative stress, e.g., that the mitochondria are producing reactive oxygen intermediates during respiration that cannot be detoxified in the absence of the peroxisomes. The second hypothesis is that some component of 2% glucose (at 22°C) or 2% acetate (at 30°C). (B) Punctate localization of DsRED-SKL is impaired in the pex5 mutant background, but not in the pex7 mutant background. (C) Growth of the strains shown in panel A on glucose or galactose (at 30°C).
primary metabolism, such as nitrogen/amino acid or carbon metabolism, is localized to the peroxisomes of *C. neoformans*.

First, the possible role of reactive oxygen detoxification by peroxisomes was examined. To test this hypothesis, we examined the four catalases in the *C. neoformans* genome. One catalase (encoded by CAT2) contains a PTS2 motif, suggesting it could be targeted to the peroxisomes in a Pex7-dependent manner. Cat2 also clusters within the peroxisomal catalase clade by phylogenetic analysis (20). The catalase CAT2 gene was mutated (20), and the cat2 mutant strain exhibited wild-type growth in the presence of glucose, in contrast to a *pex1* or *pex6* mutant, showing that this gene is not required for the phenotype. However, the *C. neoformans* quadruple catalase mutant also grows like the wild type on glucose and has no in vitro phenotype (20), and the recent finding of the absence of catalases in *N. crassa* peroxisomes suggests that this enzymatic marker may not be representative of peroxisome function (63).

The *pex1* and *pex6* mutants are no more hypersensitive to H$_2$O$_2$ than the wild type, and addition of the antioxidant ascorbic acid (vitamin C) did not improve growth of *pex* mutants in YNB medium (data not shown). Further evidence against an oxidative stress phenotype is that no change in growth was observed at 37°C, a high temperature that can trigger increased reactive oxygen species (Fig. 7B) (data not shown). Taken together, these observations suggest that the glucose-specific phenotype is not attributable to a change in oxidative stress tolerance.

Second, the role of nutrient availability was examined. Consistent with normal growth on acetate, but not glucose, growth rates on a wide selection of synthetic omission media were the same for the strains (data not shown), suggesting that poor growth was not due to a role for the peroxisomes in biosynthesis of a specific amino acid or nucleotide. Next, different carbon sources were investigated. Of four monosaccharides tested, glucose, fructose, and mannose showed similar reduced growth rates of *pex1* or *pex6* mutant strains compared to wild-type cells. In contrast, growth of the *pex1* and *pex6* mutant strains on galactose was nearly identical to that of the wild type (Fig. 7A and B). This was apparent in both solid agar and liquid cultures. All four monosaccharides enter glcyolysis to produce fructose 6-phosphate as a common intermediate. The key difference between galactose with fructose, glucose, and mannose is that the later three require hexokinase activity to enter the glycolytic pathway. Yeast extract-peptone base is a rich medium. While we observed a slight decrease in growth rate of the *pex1* and *pex6* strains on YPD medium that contains glucose, we note that *C. neoformans* exhibits robust growth on yeast extract-peptone in the absence of glucose, whereas no difference in growth was observed between the *pex* mutants and the wild type. In addition, hexokinases have various specificities towards different sugars, which may account for the slight variations in growth observed on different carbon sources (Fig. 7A).

The *C. neoformans* genome was searched for hexokinase homologs using the *S. cerevisiae* *HKX1* and *HXK2* genes and the glucose-specific glucokinase (*GLK1*) gene. Two matches were obtained: one (*HXK1*) most similar to *HXK1* and *HXK2* of *S. cerevisiae* and the other (*HXK2*) with highest similarity to *GLK1*. The predicted protein sequences of the two *C. neoformans* genes were examined for potential peroxisome targeting sequences. There is no evidence of a C-terminal PTS1-type sequence (like -SKL) for either. For *C. neoformans* Hxk2, there was an N-terminal stretch of nine amino acids (KVVVDI VKHFI) similar to the most recently described consensus for PTS2 (R/K)(L/V/I/Q)XX(L/V/I/H/Q)(L/S/G/A/K)(H/Q)(L/ A/F) (56). We hypothesized that impaired function of Hxk2 in the peroxisome mutants could be due to mislocalization of this protein to the cytoplasm, leading to inactivity or a new deleterious function.

**Hexokinase 2 (Hxk2) deletion partially suppresses the pex1 phenotype, but Hxk2 is not localized to the peroxisomes.** Based on the potential PTS sequence in hexokinase and aided by the new fluorescence protein vectors, the *HXK2* gene was studied. The *HXK2* gene was deleted, and a double *hxk2* *pex1* mutant was isolated by crosses and confirmed by Southern blot analyses (data not shown). The *hxk2* mutant showed reduced growth on glucose, but equal growth on galactose, relative to the wild-type strain (Fig. 8A and B; Table 4). However, growth in the *hxk2* mutant was not as severely reduced as that in the *pex1* mutant, providing an opportunity to assess the effects of deletion of both genes. The *hxk2* *pex1* double mutant had a growth rate like that of the *hxk2* single mutant, not the *pex1* mutant, in the presence of glucose, as well as in media containing glucose and acetate or galactose. Thus, a partial suppression of the growth defects of the *pex1* mutation by deletion.
of HXK2 suggests that the defects observed in pex1 mutants could be due to incorrect localization of Hxk2.

The HXK2 gene was fused to the DsRED or GFP genes and expressed in C. neoformans cells from a constitutively active promoter (from histone H3; fluorescence was too low from the native HXK2 promoter [data not shown]). In most cells, the protein was localized to the cytoplasm and a single structure in the cell (Fig. 8C). However, when the Hxk2-DsRED protein was expressed in a pex5 or pex1 background, no change was seen in this punctate localization (data not shown), suggesting it may be an artifact of overexpression or fusion to the DsRED protein. In further confirmation, the HXK2 gene was fused to the GFP gene and introduced into C. neoformans cells. In this case, localization was solely cytoplasmic, with no evidence of any punctate localization (Fig. 8C). Thus, we conclude that Hxk2 is not localized to the peroxisomes in C. neoformans and that the fungus therefore does not contain the equivalent of a glycosome.

**DISCUSSION**

*C. neoformans* is a fungus, pathogenic to humans, that lives in both the environment and the mammalian host. Therefore, the fungus must be able to adapt to diverse nutrient conditions to be successful. Here we investigated the role of peroxisomes in the biology of *C. neoformans*. To assess peroxisome function, two genes were selected initially for targeted mutation. Pex1 and Pex6 homologs are well studied as functionally-related AAA-type proteins with a role in the assembly of small peroxisomal vesicles into mature peroxisomes and import of proteins into peroxisomes (59). Pex1 and Pex6 interact physically in fungal and mammalian cells and function in early stages of peroxisome formation (13, 17, 32, 69, 71). Disruption of the interaction between Pex1 and Pex6 is a common pathological mechanism in patients with Zellweger syndrome, which is a class of peroxisomal defects causing early death due to accumulation of toxic intermediates usually metabolized in the peroxisome (17). A homolog of *PEX6* is essential for virulence of the fungi Colletotrichum lagenarium on bean and Magnaporthe grisea on rice or barley (36, 60). In both fungi, the *PEX6* homologs are required for fatty acid utilization, such as growth on Tween 80 or olive oil. Mutants are unable to use fatty acids during formation of the specialized infection structure, the appressorium, but can infect plants when inoculated into a wound site.
The phenotype of the *C. neoformans* *pex*1 or *pex*6 mutants includes reduced growth on fatty acids; however, the mutants can use acetate as a sole carbon source. These data suggest that, like many other organisms, peroxisomes are required for utilization of some fatty acid sources. In contrast to both plants and other fungi, the ability to utilize acetate in the *pex*1 or *pex*6 mutants suggests that glyoxylate pathway components are unlikely to be localized solely in the peroxisome or are present in

![Image](image_url)

**FIG. 8.** Hexokinase 2 is required for efficient utilization of glucose but is not localized to peroxisomes. (A) Growth of 10-fold dilutions of strains on acetate, galactose (30°C), or glucose (22°C) medium for 3 days. (B) Growth of strains in liquid yeast nitrogen base supplemented with 2% glucose, galactose, or acetate at 30°C. Error bars are the standard error of the mean (n = 3). (C) Intensity and localization of DsRED or GFP expressed from the histone H3 promoter fused or unfused to the Hxk2 protein in the wild-type background. Micrographs were overexposed (10,000 ms) for the green and red channels to highlight the lack of fluorescence or autofluorescence, except for +Hxk2-GFP (1,400-ms green channel), +DsRed (3,200-ms red channel) and +Hxk2-DsRed (1,400-ms red channel).
remnant peroxisome bodies or mislocalized (but functional) in the cytoplasm or another organelle. There is evidence that the glyoxylate pathway is localized to peroxisomes in fungi, including the ascomycetes Ashbya gossypii, Aspergillus nidulans, Candida tropicalis, Botryosphaeria dothidea, Hansenula polymorpha, and Neurospora crassa (3, 11, 29, 35, 45, 74) and in the basidiomycetes Fomitopsis paludis and Coprinus species (8, 54, 62). Thus, the peroxisomes of C. neoformans function somewhat similarly to those of S. cerevisiae, which are also not required for acetate metabolism (12) (Fig. 4). Current evidence suggests Icl1 is not localized in the peroxisomes, while Ms1 is localized there only in the presence of oleic acid (9, 38, 70). In recent studies, it has been shown that mutants lacking the PEX5 or PEX6 homologs of C. albicans or A. nidulans, respectively, can also grow efficiently on acetate (25, 57), whereas at least in another ascomycete species, C. lagenarium, mutation of the PEX6 homolog reduces growth on acetate (2). While these studies do not exclude the glyoxylate pathway enzymes from being present in the peroxisomes during β-oxidation of fatty acids, the glyoxylate pathway can function under other growth conditions in pex mutants. It is possible that the use of fatty acids as a peroxisome induction source may have biased previous reports on the localization of the enzymes in fungi.

In contrast to other fungi, C. neoformans has an unusual requirement for peroxisomes for efficient growth in the presence of monosaccharides like glucose, fructose, and mannose that are metabolized by hexokinase. Hexokinase is the first enzyme of monosaccharide metabolism with a role in glycolysis, including hexokinase, are localized in the peroxisomes (reviewed in reference 55). Mutation of either PEX2 or PEX14 in T. brucei by double-stranded RNA interference causes the organisms to die in the presence of glucose (15, 23). The proteins of C. neoformans encoding the other nine enzymes in the glycolytic pathway were examined for possible peroxisomal targeting sequences. No evidence for a PTS1 was found, and only enolase had a potential PTS2 sequence (KIDOLQIOL), although not at the N terminus where PTS2 is usually, though not invariably, located. We were excited by the prospect of C. neoformans may also have the equivalent of a glycolysis. Deletion of the HXK2 gene results in a reduced growth rate on glucose, and the hsk2 pex1 double mutants partially rescue the reduced growth rates seen in the pex1 single mutants grown in the presence of glucose. However, Hsk2 fusions to fluorescent markers were localized either in the cytoplasm or to a nonperoxisomal structure, depending on the fusion protein. Thus, we hypothesize that Hsk2 regulates a peroxisomal protein required for C. neoformans growth, either directly or via a glycolytic intermediate, such as glucose-6-phosphate, which is a signaling molecule in other fungi (48).

Our initial aim was to investigate aspects of the genetic controls of virulence of C. neoformans. Enzymes of the glyoxylate pathway (here Ms1 and previously Icl1) are dispensable for C. neoformans virulence. We were reluctant to test the virulence of the pex1 mutant towards mice because the strain showed a clear growth defect on minimal medium. Nevertheless, when tested for virulence, pex1 mutant strains were as virulent as the wild type, thus showing that (i) growth patterns on minimal medium in vitro are not reliable predictors for virulence outcome and (ii) during mammalian infection, fatty acid utilization and glucose metabolism are unlikely to be the major nutrients available for growth, in contrast to other fungi (37). It has recently been shown that the pex5 mutant of C. albicans shows virulence equivalent to that of wild-type strains in a murine infection model (57). C. neoformans associates with environmental predators, like amoeba and possibly insects. We also tested the mls1 and pex1 mutants in the wax moth system and found no reduction in virulence in this insect host. While our research aimed to identify potential new drug targets for controlling cryptococcosis, these data show that the glyoxylate pathway and peroxisomes are less than ideal targets. Future research on peroxisome function in C. neoformans, which is an outstanding system for molecular genetic analysis, will focus on the identification of the mechanisms of interaction of these organelles with glucose metabolism via proteomic or genetic analyses.

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