

Peroxisomal Fatty Acid β -Oxidation Is Not Essential for Virulence of *Candida albicans*[∇]

Katarzyna Piekarska,¹ Els Mol,¹ Marlene van den Berg,¹ Guy Hardy,¹ Janny van den Burg,¹ Carlo van Roermund,² Donna MacCallum,³ Frank Odds,³ and Ben Distel^{1*}

Departments of Medical Biochemistry¹ and Genetic Metabolic Diseases,² Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands, and Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, Aberdeen AB25 2ZD, United Kingdom³

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Phagocytic cells form the first line of defense against infections by the human fungal pathogen *Candida albicans*. Recent in vitro gene expression data suggest that upon phagocytosis by macrophages, *C. albicans* reprograms its metabolism to convert fatty acids into glucose by inducing the enzymes of the glyoxylate cycle and fatty acid β -oxidation pathway. Here, we asked whether fatty acid β -oxidation, a metabolic pathway localized to peroxisomes, is essential for fungal virulence by constructing two *C. albicans* double deletion strains: a *pex5* Δ /*pex5* Δ mutant, which is disturbed in the import of most peroxisomal enzymes, and a *fox2* Δ /*fox2* Δ mutant, which lacks the second enzyme of the β -oxidation pathway. Both mutant strains had strongly reduced β -oxidation activity and, accordingly, were unable to grow on media with fatty acids as a sole carbon source. Surprisingly, only the *fox2* Δ /*fox2* Δ mutant, and not the *pex5* Δ /*pex5* Δ mutant, displayed strong growth defects on nonfermentable carbon sources other than fatty acids (e.g., acetate, ethanol, or lactate) and showed attenuated virulence in a mouse model for systemic candidiasis. The degree of virulence attenuation of the *fox2* Δ /*fox2* Δ mutant was comparable to that of the *icl1* Δ /*icl1* Δ mutant, which lacks a functional glyoxylate cycle and also fails to grow on nonfermentable carbon sources. Together, our data suggest that peroxisomal fatty acid β -oxidation is not essential for virulence of *C. albicans*, implying that the attenuated virulence of the *fox2* Δ /*fox2* Δ mutant is largely due to a dysfunctional glyoxylate cycle.

The opportunistic human fungal pathogen *Candida albicans* is part of the normal microbial flora that colonizes mucosal surfaces, including the gastrointestinal tract and the oral and vaginal mucosa. When the immune system is compromised, superficial colonization by the fungus causes oropharyngeal thrush, vaginitis, and cutaneous infections. However, in severely immunosuppressed patients such as those who have undergone organ transplantation or chemotherapy or are taking broad-spectrum antibiotics, *C. albicans* may penetrate into deeper tissue and disseminate via the bloodstream, invading organs and causing life-threatening systemic infections (4).

The balance between benign commensal organism and invasive pathogen is largely determined by the activity of the host's innate immune system, and patients with defects in innate immunity are extremely sensitive to systemic *C. albicans* infections (18, 20, 39, 44). When phagocytic cells of the innate immune system such as macrophages and neutrophils encounter *C. albicans* cells, they take up the fungus into an intracellular compartment called the phagosome, which fuses with lysosomes to form the phagolysosome. The phagolysosome, characterized by the presence of antimicrobial compounds and reactive oxidative species and a shortage of key nutrients necessary for metabolism, is a hostile environment for *C. albicans*. However, often, in an ex vivo setting, *C. albicans* is able to survive its encounter with macrophages. Once inside, the in-

tracellular conditions of the phagolysosome trigger *C. albicans* to grow as hyphae, which puncture the plasma membrane, thereby destroying the macrophage and thus allowing the fungus to escape.

This outcome illustrates the ability of *C. albicans* to rapidly adapt to the hostile environment of the macrophage. The response of *C. albicans* to phagocytosis by macrophages has been studied by genome-wide expression profiling and differential display (15, 21). Both reports revealed that *C. albicans* reprograms its metabolism in order to cope with nutrient deprivation. In particular, the more extensive studies of Lorenz et al. (15) showed a dramatic upregulation of a specific set of metabolic pathways, i.e., gluconeogenesis, the glyoxylate cycle, and fatty acid β -oxidation, that are all required to convert fatty acids into glucose, implying that inside the macrophage the fungus utilizes lipids for energy production and biosynthesis. Similarly, following exposure to human neutrophils, *C. albicans* downregulates the glycolytic genes and activates genes encoding the key enzymes of the glyoxylate cycle (*ICL1* [isocitrate lyase] and *MLS1* [malate synthase]), indicating that the cells face a carbohydrate-poor environment (8, 24). The observation that disruption of *ICL1* resulted in a significantly attenuated virulence of *C. albicans* in a mouse model supports the notion that a functional glyoxylate cycle is required for full virulence in vivo (1, 16). A crucial role for glyoxylate cycle enzymes in virulence has also been shown for *Mycobacterium tuberculosis* (19), a bacterial pathogen of mammals; for *Leptosphaeria maculans* (11), *Magnaporthe grisea* (43), and *Stagonospora nodorum* (31), plant-pathogenic fungi; and for *Rhizoglyphus fragans* (40), a bacterial pathogen of plants. These studies

* Corresponding author. Mailing address: Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Phone: 31-20-5665127. Fax: 31-20-69915519. E-mail: b.distel@amc.uva.nl.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
SC5314		45
BWP17	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG</i>	45
YKP1	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG PEX5/pex5Δ::ARG4</i>	This study
YKP3	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex5Δ::HIS1/pex5Δ::ARG4</i>	This study
CPK1	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex5Δ::HIS1/pex5Δ::ARG4</i>	This study
CPK3	<i>ura3Δ::imm434/ura3Δ::URA3::PEX5 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG pex5Δ::HIS1/pex5Δ::ARG4</i>	This study
CPK5	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::HIS1 arg4Δ::hisG/arg4Δ::ARG4</i>	This study
CPK12	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1</i>	This study
CEM15	<i>ura3Δ::imm434/ura3Δ::URA3::FOX2 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1</i>	This study
CEM16	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG fox2Δ::ARG4/fox2Δ::HIS1</i>	This study
CMD3	<i>ura3Δ::imm434/ura3Δ his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	This study
CMD8	<i>ura3Δ::imm434/ura3Δ::URA3 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	This study
CMD6	<i>ura3Δ::imm434/ura3Δ::URA3::ICL1 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG icl1Δ::HIS1/icl1Δ::ARG4</i>	This study

imply that the glyoxylate cycle, and thus lipid metabolism, is a key factor in virulence, in both animal and plant pathogens.

In *C. albicans* and other fungi, the breakdown of fatty acids takes place only inside peroxisomes, and no mitochondrial β -oxidation is found in these organisms (10). Similarly, in most fungi the key enzymes of the glyoxylate cycle, i.e., ICL1 and MLS1, are localized in peroxisomes (17, 33, 34), although in the yeast *S. cerevisiae* either one or both enzymes may be extraperoxisomal, depending on the growth conditions (14, 32). Together, these observations suggest an important role for peroxisomes and peroxisomal metabolism in virulence of fungal pathogens.

Peroxisomes are single-membrane-bound organelles that not only contain the enzymes for the β -oxidation of fatty acids but also can be involved in a variety of other metabolic pathways, such as the inactivation of toxic substrates (H_2O_2 -based respiration), the synthesis of etherphospholipids (in mammals), and the breakdown of purines and amino acids (35). Peroxisome biogenesis is a conserved process among eukaryotes, involving the concerted action of at least 32 proteins (peroxins) that are encoded by *PEX* genes (6, 41). Most of these peroxins function in targeting and import of peroxisomal matrix proteins that are nucleus encoded and synthesized on cytosolic ribosomes. The majority of peroxisomal matrix enzymes contain a type I peroxisomal targeting signal (PTS1), while only a few contain a type II peroxisomal targeting signal (PTS2) (22). Proper targeting and import of PTS1 and PTS2 proteins is mediated by the cycling receptors Pex5p and Pex7p, respectively.

To test whether impaired function of peroxisomes can affect virulence in *C. albicans*, we constructed a mutant lacking the PTS1 receptor Pex5p. Also, we generated a mutant that has no β -oxidation activity by deleting the *FOX2* gene, encoding the second enzyme of the β -oxidation pathway. While both mutant strains were unable to grow on media with fatty acids as sole carbon sources and showed strongly reduced β -oxidation activity in vitro, only the *fox2Δ/fox2Δ* strain was attenuated in virulence to a degree comparable to that of the *icl1Δ/icl1Δ* mutant. In vitro growth assays revealed that the *fox2Δ/fox2Δ* and *icl1Δ/icl1Δ* mutants could not efficiently utilize two-carbon compounds or lactate, whereas the *pex5Δ/pex5Δ* mutant showed wild-type growth rates on these carbon sources. These rather unexpected results are discussed in the context of the

proposed role of fatty acid metabolism and the glyoxylate shunt in virulence.

MATERIALS AND METHODS

Media and culture conditions. *C. albicans* strains were grown at 28°C unless otherwise stated. For routine nonselective culturing of *C. albicans* strains, 2% Bacto peptone–1% yeast extract–2% glucose–80 μ g/ml uridine was used. *C. albicans* transformants were selected and grown on minimal solid medium containing 0.67% yeast nitrogen base (YNB) without amino acids (Difco), 2% glucose, 80 μ g/ml uridine, and amino acids (20 μ g/ml arginine and 20 μ g/ml histidine) as needed. Plates used for spot assays had the same composition and contained glucose (2%), ethanol (2%), lactate (2%), or sodium acetate (2%, pH 5.0) as a carbon source. The liquid medium used for growing cells for electron microscopy or β -oxidation measurements contained 0.5% potassium phosphate buffer (pH 6.0), 0.3% yeast extract, 0.5% peptone, 0.2% Tween 40, and 0.12% oleic acid as a carbon source. Liquid minimal oleate medium, used for growth curves, contained 0.67% YNB, 0.2% Tween 40, and 0.12% oleate. Before being shifted to one of these media, cells were grown on minimal glucose medium (0.3% glucose, 0.67% YNB) for at least 24 h.

Strains and primers. *Candida albicans* strains used in this study are listed in Table 1 and are derivatives of BWP17 (45). Primers are listed in Table 2.

The *PEX5*, *FOX2*, and *ICL1* genes were deleted using a PCR-based procedure with primers containing 60- to 70-bp regions of sequence identity to the 5' and 3' flanking sequences of the open reading frames (ORFs) (45). The technique involved the construction of two cassettes. The first one, containing the *ARG4* auxotrophic marker, was amplified from the plasmid pRS-Arg4*SpeI by using primers CaDPex5F and CaDPex5R (*PEX5* disruption), FOX2-F1 and FOX2-R1 (*FOX2* disruption), or S11-ICL1 and S22-ICL1 in combination with extension primers E11-ICL1 and E22-ICL1 (*ICL1* disruption). The second cassette contained the *HIS1* auxotrophic marker, which was amplified on plasmid pGEM-HIS1 with the same primers as used for amplification of the *ARG4* cassette. Gene deletions were created through sequential rounds of transformation of strain BWP17 with the disruption cassettes.

To construct pKP5, plasmid pRSArg-N/Ura (45) was digested with BamHI and SpeI, releasing a 1,970-bp fragment encompassing the *ARG4* gene. The fragment was cloned between the BamHI and SpeI sites of pAN1 (13), resulting in plasmid pKP5, which was used for complementation of the arginine auxotrophy of BWP17. Plasmid pKP6, used for complementation of the *pex5Δ/pex5Δ* mutant with the *PEX5* gene, was constructed as follows. The wild-type *PEX5* sequence from nucleotide –842 to +2374 was amplified from genomic DNA with primers pex5intF and pex5intR, carrying XhoI and BamHI restriction sites, respectively, and ligated into pGEM-T Easy (Promega) vector. The resulting plasmid, pKP1, was digested with XhoI, and the overhanging ends were filled in using Klenow fragment (25). The linearized plasmid was recovered and digested with BamHI, and the fragment encompassing the *PEX5* gene was cloned into plasmid pLUBP (23) digested with BamHI and StuI. The resulting plasmid, pKP6, was digested with PacI and integrated into the *URA3* locus of strain YKP3 (*pex5Δ/pex5Δ*), giving rise to the reintegrant strain CPK3 (*pex5Δ/pex5Δ* + *PEX5*). The wild-type *ICL1* sequence from nucleotide –590 to +2016 was amplified from genomic DNA of strain SC5314 with primers ICL1-PstI and Sac1-ICL1 and ligated into vector pGEM-T (Promega). After sequencing, the 2.6-kb fragment

TABLE 2. Primers used in this study

Primer	Sequence (5'→3')
CaDPex5F.....	TAATTTTGGATTTTCGAAAAATTTGTTATTTT TCTTTCTCCCATATCATTATCAAAGAATG TGGAATTTGGAGCGGATA
CaDPex5R.....	ACAGCTAGAAAATAAATAATTTGCAATTTT GCCATAAATACATACCTATATACTTAGA AGTTTTCCAGTCACGACGTT
CaPex5F.....	CCACTTATCATTTCTCGGTGG
CaPex5R.....	GTGGTGGTAGGAAGCGTTG
pex5IntF.....	CCGCTCGAGCGGCACAATGCTTGTGTA TCTG
pex5IntR.....	CGGGATCCCGTCTGAGTAAAGTTAG ACTC
URA3INTF.....	ATCAGTAGCATCATCTCTCAGCG
URA3INTR.....	TAGTGATCACTTCTCTACTCCG
pex5-F2.....	AGAGCGTTGATGACTTGGC
pex5-R2.....	GCCTTGTGTGCAACTCCTCTA
Ca arg3.....	TGCCACTGATCCATTGATGGATT
Ca arg5.....	TTGTGGCATCAATGAAGAACCAG
Ca his5.....	AAGTTCAGCAGATGGCGAGTA
Ca his3.....	GGCCAGTTCCCTTAGATCTAGA
HIS1CHR2.....	GCGACAAAGTGGTATTGACA
HIS1CHR.....	AGGTTTCCCGACTGGAAAGC
FOX2-F1.....	TTCTTTTAAATCCTTTGAAATATTTATTTT ATTAACCTCAGACATAACTTTCCCTCC CCCCCATTTGTGGAATTTGTGAGCG GATA
FOX2-R1.....	CTTAAAAAGAAAAATAAATACTAACA AAACAAAATAATATAAAAACTAAATGG CAATTTACTGCGTTTTCCAGTCACGA CGTT
FOX2-F11.....	CCCCCTTTTCTTATTTCCA
FOX2-R12.....	CAAGACAAAACCTAACATCAAAACTCA
FOX-F6.....	CCCCCCCATTATGTCCTCAA
FOX-F8.....	CAGTGTCATCGTTTCTTTCCA
S11-ICL1.....	TTTCTTTTTTCTTCTTCTCTATACTTATA CCTTTTATTCTAATAAATAAAGTAATA AACATTAATAATATCTACCGAAGCTCGT ACGCTGCAGGTC
S22-ICL1.....	CAACAGAATAACTTCTTATAATTTTACTT ACAAATTTGACTACAAAAACAGTTTTC AAATATAAATAATGTTGAATAATCTGAT ATCATCGATGAATTCGAG
E11-ICL1.....	TTTCTTTTTTTTAAATACCTTTTTTCTTTTCT TTTTT
E22-ICL1.....	TCTCTTTGACGAATAATTGCCAACAGAATA ACTTTCTT
FP-Icl1.....	TCGTTACTCCAAGTGGTCAATG
RP-Icl1.....	TTTAAATGTGCGATGGGTTCA
Sac1-Icl1.....	AAGAGCTCCCGTCGACAAAAAGAAAAA
ICL1-PstI.....	TTCTGCAGAAGGCTCAAATGTTCCCGA
Arg2.....	AATGGATCAGTGGCACCCTGGT
Arg4.....	GTAGATATTTGGTCAGTTATCTGG
His4.....	GAAATGGCCTCCCTACCACAG
His5.....	GGACGAATGAAGAAAGCTGG

encompassing the *ICL1* gene and promoter was cloned into plasmid pLUBP digested with *SacI* and *PstI*. The resulting plasmid, pEM311, was digested with *PacI* and integrated into the *URA3* locus of strain CMD3 (*icl1Δ/icl1Δ*), giving rise to reintegrant strain CMD6 (*icl1Δ/icl1Δ + ICL1*).

To isolate the *FOX2* gene, *XhoI*- and *PstI*-digested genomic DNA of *C. albicans* SC5314 was size fractionated on an agarose gel, and 4- to 5-kb fragments were isolated and cloned into vector pSP73 (Promega). A small library of 8,000 transformants was screened with a 2.6-kb *FOX2* probe that was generated by PCR on genomic DNA by using primers FOX-F6 and FOX-R8. Four independent transformants that contained a 4.5-kb *XhoI*-*PstI* fragment encompassing the *FOX2* ORF, 1.6-kb 5' sequences, and 0.3 kb of 3' sequences were identified. One of these plasmids (pEM501) was digested with *XhoI*, blunt ended, and digested with *PstI*. The resulting 4.5-kb *FOX2* fragment was inserted into pLUBP digested with *StuI* and *PstI* to give plasmid pEM503. The *FOX2* reintegrant strain CEM15 was constructed by integrating *XhoI*-digested pEM503 into strain CPK12 (*fox2Δ/fox2Δ*).

To create prototrophic strains of *pex5Δ/pex5Δ* (YKP3), *fox2Δ/fox2Δ* (CPK12), and *icl1Δ/icl1Δ* (CMD3), the strains were transformed with *PacI*- or *XhoI*-digested pLUBP, resulting in strains CPK1, CEM16, and CMD8, respectively.

Finally, a prototrophic BWP17 strain (CPK5) was constructed by sequential transformation of strain BWP17 with plasmids pLUBP, pKP5, and pGEM-HIS1 digested with *PacI*, *NotI*, and *PstI*, respectively. All strains were verified by PCR and Southern hybridization.

Southern analysis. Southern blotting was carried out with standard protocols (25), using randomly primed probes. The *PEX5* probe was a 0.7-kb *SalI*-*BamHI* fragment of plasmid pKP1 comprising 0.1 kb of the *PEX5* ORF and 0.6 kb of 3' sequences. The *FOX2* probe was a 1.7-kb *XbaI*-*ClaI* fragment comprising 0.5-kb promoter sequences and 1.2 kb of the *FOX2* ORF. Radioactive signals were revealed by exposure to Kodak Bio Max MR film (Amersham Biosciences).

Transformation. *C. albicans* was transformed using a modified lithium acetate protocol (42). The heat shock was carried out at 44°C for 15 min.

Fatty acid β -oxidation measurements. Measurements of β -oxidation in intact cells were performed essentially as described by van Roermund et al. (37) except that the cells were resuspended at an optical density (OD) at 600 nm of 1 and the incubations with radiolabeled fatty acids were allowed to proceed for 45 min. The β -oxidation capacity of wild-type cells grown on oleate in each experiment was taken as a reference (100%).

Antibodies. Polyclonal antibodies directed against *S. cerevisiae* 3-ketoacyl coenzyme A (3-ketoacyl-CoA) thiolase or catalase were used in this study. Both antibodies cross-react specifically with the corresponding peroxisomal proteins in *C. albicans* (our unpublished results).

Electron microscopy. Oleate-induced cells were fixed with 2% (wt/vol) formaldehyde, and ultrathin sections were prepared as described previously (9). Immunolabeling was performed using antibodies directed against *S. cerevisiae* 3-ketoacyl-CoA thiolase or catalase and gold-conjugated protein A.

Virulence studies. Virulence assays were performed at the University of Aberdeen, using a murine tail vein injection model. Four independent experiments were carried out with 6 to 12 female BALB/c mice in each injection group. All *C. albicans* strains, except for the *icl1Δ/icl1Δ* mutant and *icl1Δ/icl1Δ* reintegrant, were tested at least twice. Strains were grown overnight at 30°C in 1.0% neopeptone–0.4% glucose–0.1% yeast extract to early stationary phase (2×10^7 CFU/ml $\pm 0.3 \times 10^7$ CFU/ml). Cells were centrifuged (10 min at $2,500 \times g$), washed twice, and resuspended in sterile saline. The OD of the final suspension was adjusted according to a predetermined calibration curve of OD versus CFU per ml to yield suspensions of cells calculated to give suitable yeast inoculum doses for intravenous injection in 100- μ l volumes for mice weighing 20 g. Actual CFU/ml in inoculum suspensions were determined by viable counting procedures and ranged from 1.6×10^4 to 4.5×10^4 CFU per gram body weight. Animals were observed and weighed daily for a period of 28 days after challenge. Animals that lost more than 20% of their body weight or otherwise showed signs of irreversibly severe disease were humanely killed and recorded as having died on the following day. Tissue burdens were assessed on the day of demise of the animal. For all animals, the left and right kidneys and brain were removed postmortem, weighed, and homogenized in saline, and tissue burdens of fungi were determined by viable counts of the homogenate. For the purpose of calculating mean burdens, when tissues were negative for recovery of *C. albicans* they were recorded as containing 0.5 log₁₀ CFU/g below the detection threshold, i.e., 1.8 log₁₀ CFU/g for kidney and 1.3 log₁₀ CFU/g for brain.

Statistical analysis. Survival data were analyzed by log rank/Kaplan-Meier statistics. Tissue burden data were analyzed by the Mann-Whitney *U* test.

RESULTS

Identification of the *C. albicans* *PEX5* and *FOX2* genes. In order to identify the genes encoding CaPex5p and CaFox2p, a BLASTp search with *S. cerevisiae* Pex5p and Fox2p was performed. This resulted in the identification of a single Pex5-like open reading frame (orf19.5640), encoding a protein of 593 residues, and a single Fox2-like open reading frame (orf19.1809), encoding a 907-amino-acid protein. Sequence alignment of CaPex5p and CaFox2p with their *S. cerevisiae* homologues revealed overall identities of 41% and 52%, respectively. Accordingly, these ORFs were named CaPEX5 and CaFOX2. The *PEX5* gene was amplified by PCR with genomic DNA prepared from the wild-type strain SC5314, using gene-specific oligonucleotides. The clone was sequenced to confirm its identity. Because of difficulties with PCR amplification of the 5' end of the *FOX2* gene, a small genomic library was constructed

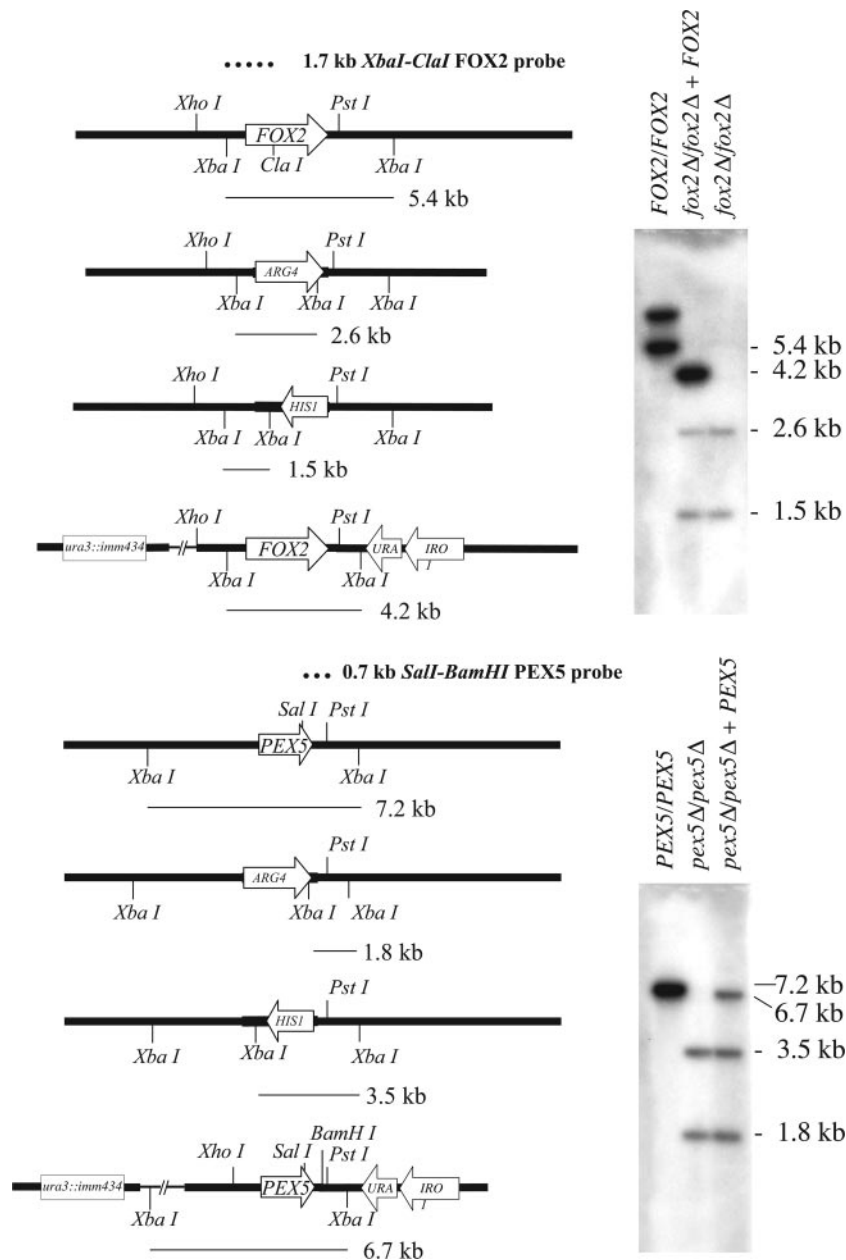


FIG. 1. Southern blot analysis of the constructed strains. Shown are the genomic configurations of the *PEX5* and *FOX2* wild-type loci, the deleted derivatives, and the reintegrants at the *URA3* locus. Genomic DNA was digested with *XbaI* and analyzed by Southern blotting using the indicated probes (dotted lines). The strains used were the prototrophic wild-type strain CPK5 (*PEX5/PEX5* and *FOX2/FOX2*), CPK1 (*pex5Δ::ARG4/pex5Δ::HIS1*), CPK3 (*pex5Δ::ARG4/pex5Δ::HIS1* + *PEX5*), CEM16 (*fox2Δ::ARG4/fox2Δ::HIS1*), and CEM 15 (*fox2Δ::ARG4/fox2Δ::HIS1* + *FOX2*). Due to allelic variation, resulting in loss of the *XbaI* site downstream of the *FOX2* gene, two bands are visible for the wild-type strain CPK5 probed with the *FOX2* probe.

(see Materials and Methods), and a single clone containing a 4.6-kb fragment was isolated, encompassing 1.6-kb upstream sequences, the complete *FOX2* ORF, and 0.3-kb downstream sequences.

Construction of *C. albicans pex5* and *fox2* null mutants by targeted gene disruption. To investigate the roles of CaPex5p and CaFox2p in *C. albicans* virulence, we created strains in which both copies of either the *PEX5* or the *FOX2* gene were deleted (*pex5Δ/pex5Δ* and *fox2Δ/fox2Δ*) by using a PCR-based gene disruption procedure described by Wilson et al. (45).

Correct integration of the *ARG4* and *HIS1* cassettes at the *PEX5* and *FOX2* loci was verified by PCR (results not shown) and Southern blot analysis (Fig. 1).

Recently, it was demonstrated that different genomic positions of the *URA3* gene might result in different levels of *URA3* gene expression, thereby altering the virulence potential of *C. albicans* strains (2, 5). To avoid positional effects on *URA3* expression, we reintroduced the *URA3* gene at a common locus in all strains. For this, we used vectors based on the pLUBP plasmid (23), which contains the complete *URA3* gene and the

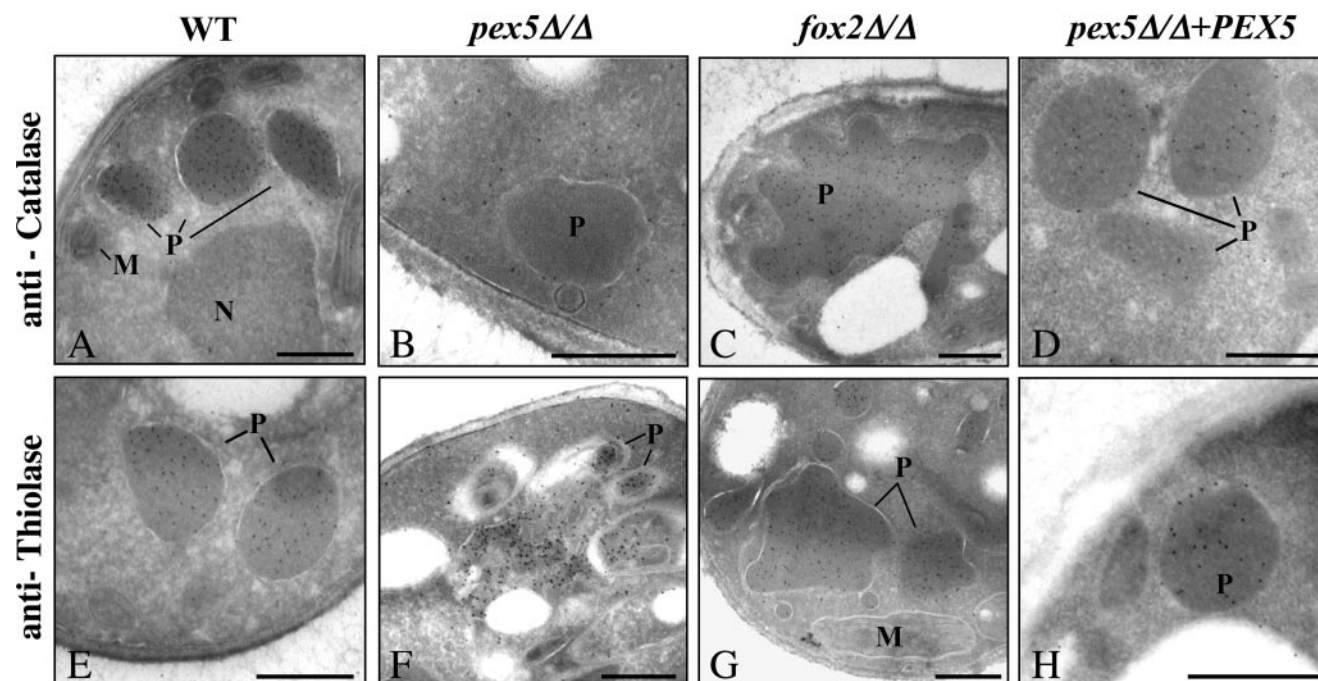


FIG. 2. Characterization of the *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* strains by immunoelectron microscopy. Oleate-induced wild-type cells (A and E), *pex5Δ/pex5Δ* cells (B and F), *fox2Δ/fox2Δ* cells (C and G), or complemented *pex5Δ/pex5Δ* cells (D and H) were fixed and prepared for immunocytochemistry. Cryosections were incubated with antibodies directed against catalase (A to D) or thiolase (E to H), and antigens were visualized with immunogold particles conjugated to protein A. P, peroxisome, M, mitochondrion, N, nucleus. Bars, 0.5 μ m.

flanking *IRO1* gene. Vectors were integrated at the *ura3* locus, thereby restoring Ura3 prototrophy and reintroducing the wild-type copy of the deleted gene at the same locus (Fig. 1). Ura3 prototrophs of the double-deletion strains were obtained by integration of an “empty,” linearized pLUBP vector. The correct integration of plasmids was confirmed by PCR and Southern analysis (results not shown).

Characterization of the *pex5* and *fox2* null mutants by immunoelectron microscopy. Previous studies with other organisms have shown that deletion of the *PEX5* gene, encoding the receptor Pex5p, results in mislocalization of peroxisomal proteins containing a PTS1, while PTS2 proteins are properly localized.

To determine whether the *Capex5Δ/pex5Δ* strain also shows a PTS1-dependent mislocalization of peroxisomal matrix proteins, we performed immunoelectron microscopy on oleate-induced *C. albicans* cells with antibodies directed against *S. cerevisiae* thiolase (a PTS2 protein) and catalase (a PTS1 protein). Western blot analysis of total lysates of wild-type *C. albicans* cells showed that both antibodies specifically cross-react with the corresponding proteins in *C. albicans* (data not shown). Immunocytochemistry of the *pex5Δ/pex5Δ* strain revealed mislocalization of catalase as indicated by significant labeling of the cytosol and nucleus, whereas thiolase was confined to membrane structures (Fig. 2). These results are in line with previous studies of *S. cerevisiae*, which showed that in *pex5Δ/pex5Δ* cells, thiolase could still be imported in peroxisomal membrane structures lacking all of the PTS1-containing matrix proteins (36). In wild-type *C. albicans* cells, the anti-catalase and antithiolase antibodies only labeled peroxisomal profiles, confirming the specificity of the antibodies used. Catalase

and thiolase labeling in the complemented *pex5Δ/pex5Δ* strain (*pex5Δ/pex5Δ* + *PEX5*) was indistinguishable from that in wild-type cells, indicating that PTS1 import was fully restored in the revertant.

As expected, deletion of the *FOX2* gene, encoding the bi- or multifunctional enzyme (Fox2p), did not influence the transport of peroxisomal profiles, as indicated by the strong labeling of the peroxisomal profiles with both anticatalase and antithiolase. However, peroxisomes in *fox2Δ/fox2Δ* cells appeared to be larger and more irregular in structure than those in wild-type cells (or the complemented *fox2Δ/fox2Δ* strain [data not shown]). Such a phenotype has been observed before in yeast and mammalian mutants with deficiencies in peroxisomal fatty acid β -oxidation and has been attributed to defects in peroxisome proliferation (29, 38).

The *pex5* and *fox2* null mutants are unable to grow on oleic acid-containing medium. *S. cerevisiae* mutants with defects in peroxisome biogenesis (e.g., *pex5Δ*) or peroxisomal β -oxidation (e.g., *fox2Δ*) cannot utilize fatty acids as the sole carbon source (10). Therefore, we tested the ability of the *C. albicans* *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* null mutants to grow on minimal medium containing oleic acid as the exclusive carbon source (Fig. 3). No increase in optical density was observed for the *pex5Δ/pex5Δ* or the *fox2Δ/fox2Δ* strain during the time course of the experiment (24 h). In contrast, the strains with the reintroduced *PEX5* or *FOX2* gene showed growth rates comparable to that of the wild-type strain. All strains grew equally well on medium containing glucose as the carbon source (see Fig. 6). From these experiments, we conclude that both deletion strains cannot use oleic acid as the sole carbon source and

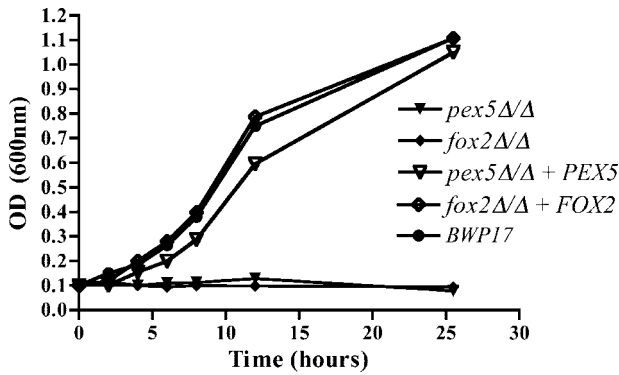


FIG. 3. The *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* mutants are unable to grow on oleic acid-containing medium. Strains were pregrown on minimal medium containing 0.3% glucose and inoculated at an OD at 600 nm of 0.1 in minimal oleate medium. Growth was monitored over a period of 24 h by measuring the optical density of the cultures.

that the oleic acid-nonutilizing phenotype was fully suppressed in the complemented null strains.

The *pex5* and *fox2* null mutants have strongly reduced fatty acid β -oxidation activity. The above results suggested that the *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* strains could not metabolize oleic acid. To directly determine their capacity to degrade fatty acids, we measured the oxidation of 1-¹⁴C-labeled oleic acid in these strains. As shown in Fig. 4, oxidation of [1-¹⁴C]oleic acid in the *pex5Δ/pex5Δ* mutant was strongly reduced, to about 5% of the β -oxidation activity present in wild-type cells. In the strain lacking Fox2p, β -oxidation activity dropped to background levels (\sim 0.2% of the activity in wild-type cells). Complementation of the deletion strains with the corresponding wild-type gene almost completely restored fatty acid oxidation to wild-type levels. Similar results were found when the

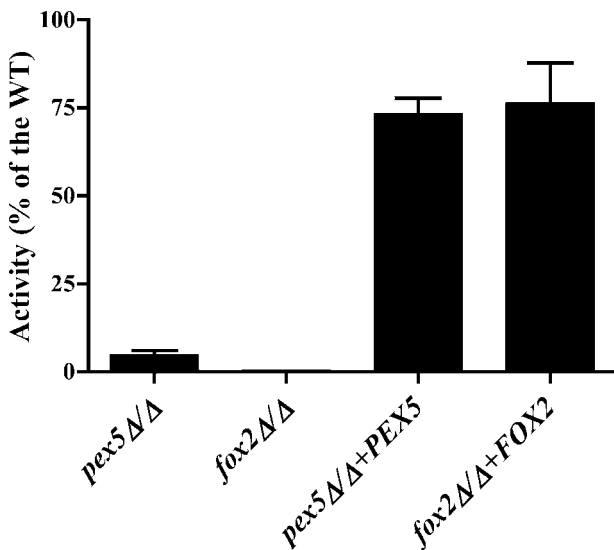


FIG. 4. The *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* mutants have strongly reduced fatty acid β -oxidation activity. Strains were induced on oleate, and β -oxidation activity was measured as described in Materials and Methods. β -Oxidation rates are expressed as percentages of the activity measured in the prototrophic wild-type (WT) strain BWP17. Error bars indicate standard deviations.

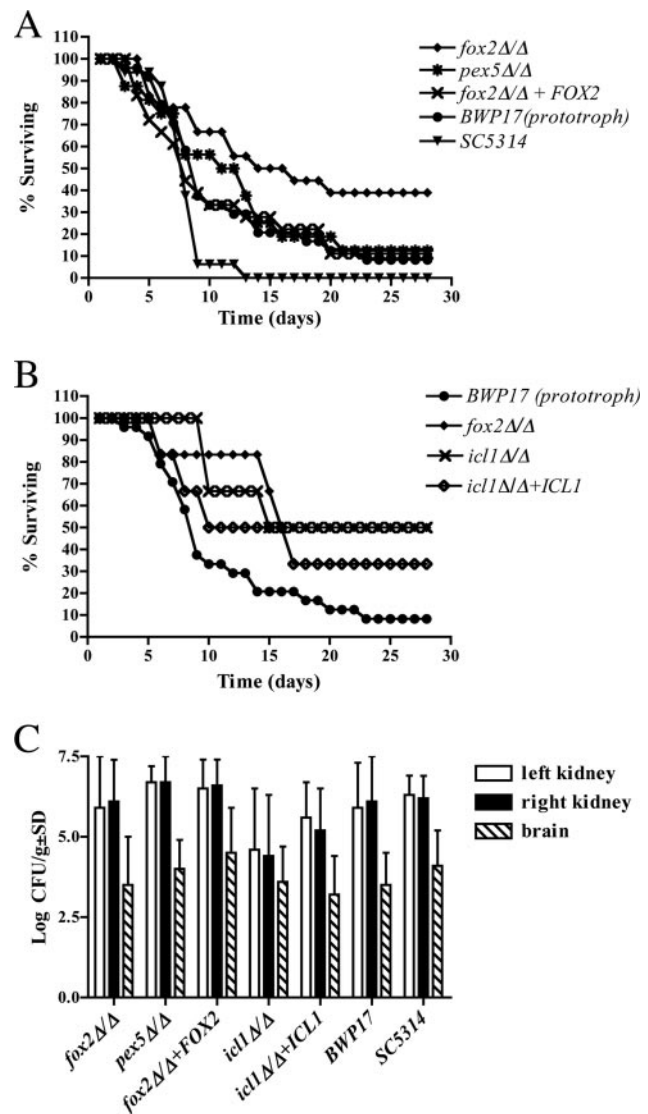


FIG. 5. Virulence of the *pex5Δ/pex5Δ*, *fox2Δ/fox2Δ*, and *icl1Δ/icl1Δ* mutants in a mouse model for systemic candidiasis. (A) Survival of the *pex5Δ/pex5Δ*, *fox2Δ/fox2Δ*, and *fox2Δ/fox2Δ* reintegrand was compared to that of the prototrophic BWP17 strain and the clinical isolate SC5314. BALB/c mice were infected intravenously with a challenge dose in the range of 1.6×10^4 to 4.5×10^4 CFU/g body weight. The combined data from four separate experiments are shown. (B) Comparison of the virulence of the *icl1Δ/icl1Δ* mutant and the *icl1Δ/icl1Δ* reintegrand with that of the *fox2Δ/fox2Δ* mutant. As a control, the survival curve of the prototrophic parental strain BWP17 is shown. (C) Mean tissue burdens in brain, left kidney, and right kidney for mice infected with each of the seven *C. albicans* strains. Error bars indicate standard deviations.

strains were tested for their ability to oxidize short-chain fatty acids (octanoic acid) (data not shown). Together, these data indicate that peroxisomal fatty acid β -oxidation of both short- and long chain fatty acids is strongly reduced in the *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* strains. Furthermore, these results support the notion that there is a single *FOX2* gene encoding the peroxisomal multifunctional enzyme in *C. albicans*.

The *fox2* null mutant, but not the *pex5* null mutant, is significantly attenuated in virulence. To assess the virulence of

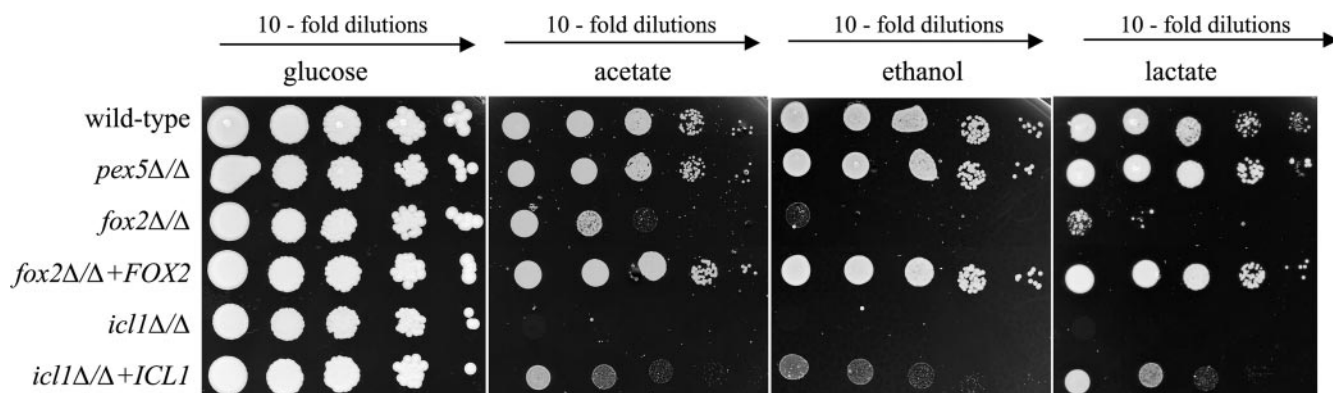


FIG. 6. Growth characteristics of wild-type and mutant strains on different carbon sources. Cells were pregrown on medium containing 0.3% glucose, washed, resuspended to a concentration of about 2.7×10^7 cells/ml, and serially diluted (1:10 dilutions). Five microliters of each dilution was spotted onto agar plates. The pictures were taken after 3 days (glucose) or five days (acetate, ethanol, and lactate) of incubation at 28°C.

the *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ* strains, four separate experiments were performed in which groups of 12 (first experiment) or 6 (second, third, and fourth experiments) female BALB/c mice were infected intravenously with a challenge dose in the range of 1.6×10^4 to 4.5×10^4 CFU/g body weight. As controls, the complemented derivatives (*fox2Δ/fox2Δ* + *FOX2* and *pex5Δ/pex5Δ* + *PEX5*), the prototrophic BWP17, and the clinical isolate SC5314 were used. In Fig. 5A the combined data from four separate experiments are summarized. The survival curves show that all strains derived from BWP17 (including the prototrophic BWP17) are slightly attenuated in virulence relative to the wild-type strain SC5314. The reason for the reduced virulence of BWP17-derived strains is currently not known, but it may be related to the observation that this strain does not have a full-length copy of chromosome 5 (27). Nevertheless, survival of the BWP17-derived *pex5Δ/pex5Δ* mutant was not significantly different from that of the prototrophic BWP17 strain ($P = 0.655$) or the *pex5Δ/pex5Δ* reintegrant (data not shown). A small but significant difference in survival between the *fox2Δ/fox2Δ* mutant and BWP17 ($P = 0.027$) was observed. Reintroduction of the *FOX2* gene completely suppressed this virulence defect, indicating that *FOX2* function is required for full virulence of *C. albicans*. Analysis of left and right kidney burdens and brain burdens of the infected mice showed no significant difference between the six strains tested (Fig. 5C and data not shown).

The *fox2* and *icl1* null mutants display similar degrees of virulence attenuation. Based on transcription profiling studies, it has been suggested that the ability of *C. albicans* to convert fatty acids into glucose, a metabolic process that requires the glyoxylate cycle, may contribute to its virulence in vivo (15, 16). Indeed, inactivation of the *ICL1* gene, encoding a key enzyme of the glyoxylate cycle, attenuated virulence of *C. albicans* (1, 16). However, since the *ICL1* gene deletions were made in different strain backgrounds by different procedures, a direct comparison with our BWP17-derived *fox2Δ/fox2Δ* strain was not possible. Therefore, we generated an *icl1* null mutant in BWP17 and determined the effect of inactivating *ICL1* on *C. albicans* virulence (Fig. 5B). Deletion of *ICL1* partially but significantly attenuated virulence ($P = 0.027$), confirming previous results (1, 16). This virulence defect was suppressed,

albeit incompletely, by reintroduction of the *ICL1* gene. Interestingly, the *icl1* null mutant displayed a similar degree of virulence attenuation as the *fox2Δ/fox2Δ* mutant. At first sight these results seem to be in concordance: *icl1Δ/icl1Δ* cells (16) and *fox2Δ/fox2Δ* cells (Fig. 3) cannot grow in vitro on fatty acids and show similar reductions in virulence, suggesting that the capacity to β -oxidize fatty acids plays a role in *C. albicans* virulence. However, our results with the *pex5Δ/pex5Δ* mutant do not support this conclusion: *pex5Δ/pex5Δ* cells also have a strongly reduced fatty acid β -oxidation activity and, accordingly, do not grow on media with fatty acids, but virulence is not affected in this mutant. We therefore investigated the growth behavior of all three mutant strains on nonfermentable carbon sources other than fatty acids.

The *fox2Δ/fox2Δ* and *icl1Δ/icl1Δ* mutants, but not the *pex5Δ/pex5Δ* mutant, display severe growth defects on nonfermentable carbon sources other than fatty acids. Serial dilutions of cells pregrown in 0.3% glucose medium were spotted onto plates containing acetate (C_2), ethanol (C_2), lactate (C_3), or glucose (C_6) as a carbon source (Fig. 6). As expected, the *icl1Δ/icl1Δ* mutant did not grow on acetate or ethanol and, surprisingly, also did not grow on lactate. The growth behavior of the *fox2Δ/fox2Δ* strain was remarkably similar to that of the *icl1Δ/icl1Δ* strain: it had strongly reduced growth on acetate or lactate and virtually no growth on ethanol. These phenotypes were suppressed completely (*FOX2*) or partially (*ICL1*) by introduction of the corresponding wild-type gene. In contrast, the *pex5Δ/pex5Δ* mutant exhibited growth rates comparable to those of wild-type cells on all three nonfermentable carbon sources. Furthermore, all strains grew equally well on glucose. These results indicate that the *fox2Δ/fox2Δ* mutant, like the *icl1Δ/icl1Δ* mutant, cannot efficiently utilize nonfermentable carbon sources such as acetate, ethanol, or lactate, a phenotype that is not observed for the *pex5Δ/pex5Δ* mutant.

DISCUSSION

Recent studies have suggested that the ability of *C. albicans* to convert fatty acids into glucose may contribute to its virulence in vivo (15, 16, 21). Three metabolic pathways are essential for this conversion: (i) fatty acid β -oxidation, a metabolic

pathway that is localized in peroxisomes in all yeasts and mediates the breakdown of fatty acids to acetyl-CoA (C_2) units; (ii) the glyoxylate cycle, a pathway that allows the conversion of acetyl-CoA units to C_4 compounds; and (iii) gluconeogenesis, for the synthesis of C_6 compounds (e.g., glucose) from C_4 units. Here we used two *C. albicans* mutants, *pex5Δ/pex5Δ* and *fox2Δ/fox2Δ*, to directly address the role of peroxisomal function and peroxisomal fatty acid β -oxidation in the virulence of this organism. The *pex5Δ/pex5Δ* mutant is disturbed in peroxisome biogenesis and specifically mislocalizes PTS1-containing proteins to the cytosol (Fig. 2). As a consequence, fatty acid β -oxidation is strongly reduced, and *pex5Δ/pex5Δ* cells cannot grow on media with oleic acid as the sole carbon source (Fig. 3 and 4). The *fox2Δ/fox2Δ* mutant lacks in vitro β -oxidation activity and, accordingly, does not grow on oleic acid-containing medium (Fig. 3 and 4). These data and the Southern blot analyses show that *C. albicans* contains a single *PEX5* gene and a single *FOX2* gene.

Despite the inability of both mutants to grow on fatty acids as the sole carbon and energy source, virulence assays in a mouse model for systemic candidiasis revealed that only the *fox2Δ/fox2Δ* strain is significantly attenuated in virulence (Fig. 5). How can the difference between the *fox2Δ/fox2Δ* mutant and the *pex5Δ/pex5Δ* mutant be explained? Direct comparison of the two mutant strains with the *icl1Δ/icl1Δ* glyoxylate cycle mutant with respect to their growth and virulence phenotypes revealed a striking similarity between the *fox2Δ/fox2Δ* mutant and the *icl1Δ/icl1Δ* mutant: both mutant strains could not efficiently utilize nonfermentable carbon sources such as acetate, ethanol, or lactate, and in a mouse model for systemic candidiasis the two mutant strains displayed similar degrees of virulence attenuation (Fig. 5 and 6). In contrast, the *pex5Δ/pex5Δ* mutant showed wild-type growth rates on these nonfermentable carbon sources and, as mentioned above, was not attenuated in virulence. These data suggest a strong correlation between in vivo virulence attenuation and the inability of mutant cells to grow in vitro on nonfermentable carbon sources other than fatty acids, and they imply that in the *fox2Δ/fox2Δ* mutant the glyoxylate cycle does not function optimally.

In *C. albicans*, like in most fungi, the key enzymes of the glyoxylate shunt are localized in the peroxisomal matrix, and their import into the organelles is dependent on the PTS1 receptor Pex5p (unpublished data). The fact that growth of the *pex5Δ/pex5Δ* strain on two-carbon compounds is not disturbed indicates that the glyoxylate cycle functions equally well in the peroxisome and in the cytosol and implies that there is no requirement for a peroxisomal compartmentalization of this metabolic pathway. Why the glyoxylate cycle is deficient in the *fox2Δ/fox2Δ* strain remains to be investigated.

Our observations are rather unexpected in light of the accumulating data that suggest an important role of the fatty acid β -oxidation pathway in bacterial (19) and fungal (15, 16, 21) virulence. The persistence defect of *M. tuberculosis* isocitrate lyase mutants is thought to be due to an inability to utilize fatty acids as the carbon source when the bacteria are in a lipid-rich environment of macrophages (19). A similar suggestion has been put forward to explain the reduced virulence of a *C. albicans icl1Δ/icl1Δ* mutant (16). In support of this, Lorenz et al. (15) found that upon ingestion by macrophages, *C. albicans* induces most of the enzymes required for the breakdown of

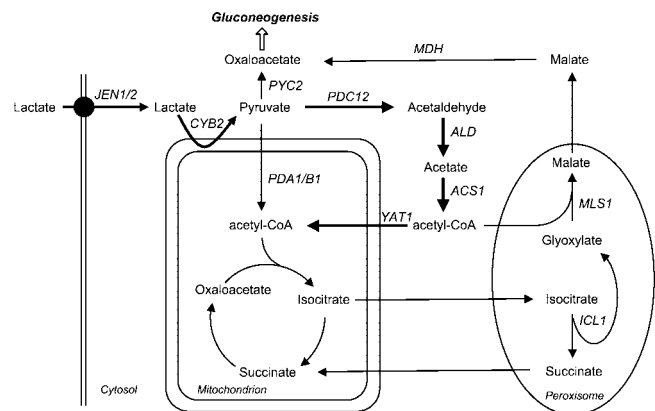


FIG. 7. Schematic drawing of metabolic routes possibly involved in lactate utilization in *C. albicans*. The figure shows the lactate transporters JEN1 and JEN2, the L-lactate dehydrogenase CYB2, the pyruvate decarboxylase pathway or “pyruvate dehydrogenase bypass” (highlighted with thick arrows), the tricarboxylic acid cycle, and the glyoxylate bypass. Other abbreviations: PDC, pyruvate decarboxylase; PYC, pyruvate carboxylase; PDA/B, pyruvate dehydrogenase complex; ALD, acetaldehyde dehydrogenase; ACS, acetyl-CoA synthase; YAT, carnitine acetyltransferase; ICL, isocitrate lyase; MLS, malate synthase; MDH, malate dehydrogenase. For details see text.

fatty acids. Our results suggest that fatty acids are not the exclusive source of acetyl-CoA within the phagolysosome. Therefore, the question arises as to which other compounds could serve as precursors of these C_2 units. Our in vitro growth assays and the transcription profiling data of Lorenz et al. provide a possible answer. Phagocytosis of both *S. cerevisiae* and *C. albicans* by macrophages results in a strongly increased expression of *ACS1* (acetyl-CoA synthase) (8.7- and 6.1-fold induced, respectively) and *YAT1* (carnitine acetyltransferase) (36.6- and 20.4-fold induced, respectively). In *S. cerevisiae*, *YAT1* is even more highly induced than the glyoxylate cycle genes (16). *ACS1* is also upregulated together with the glyoxylate cycle genes *ICL1* and *MLS1* upon incubation of *C. albicans* with neutrophils (8). The *ACS1* product, a cytosolic enzyme in yeasts (12, 28), catalyzes the ATP-dependent activation of acetate to acetyl-CoA. The cytosolic acetyl-CoA is either shuttled into mitochondria, requiring the carnitine acetyltransferase *YAT1* localized in the outer mitochondrial membrane (26), or serves as a building block for lipid synthesis (7). However, it is unlikely that acetate is present in sufficiently high concentration inside the phagolysosome to sustain growth of *C. albicans*.

A more likely explanation is that the intracellular acetate is the product of lactate degradation (Fig. 7). Among the *C. albicans* genes that are most highly induced in macrophages are *JEN1* (5.5-fold induced) and its close homolog *JEN2* (orf19.5307) (159.5-fold induced), encoding lactate permeases, and *CYB2* (15.1-fold induced), coding for L-lactate dehydrogenase (30). L-Lactate is abundantly present in the human body and is produced at high rates by red blood cells, by brain, and by muscle (3). Lactate taken up by *C. albicans* is oxidized to pyruvate by lactate dehydrogenase. The observed induction of the cytosolic pyruvate decarboxylase pathway (involving pyruvate decarboxylase [*PDC12*] [1.4-fold induced] and acetaldehyde dehydrogenase [orf19.6306] [7.6-fold induced]) and the downregulation of the mitochondrial pyruvate dehydrogenase

complex (*PDA1* and *PDB1*) (4- to 5-fold repressed) suggest that the lactate-derived pyruvate is converted to acetate in the cytosol. Strikingly, the enzymes required for lactate utilization and production of cytosolic acetyl-CoA are strongly repressed (>10-fold) in *C. albicans* challenged with fatty acids only (our unpublished microarray data). These data, together with the observation that lactate induces the expression of the lactate permease gene *JEN1* (30), may point to the presence of lactate in the microenvironment of the phagosome, the utilization of which may contribute to the survival of *C. albicans*. The requirement for the glyoxylate bypass when lactate (a C_3 compound) is present as a carbon and energy source is rather unexpected, since the lactate-derived pyruvate can be converted to oxaloacetate (C_4) by pyruvate carboxylase (*PYC2*) (Fig. 7). However, our in vitro growth assays show that the *C. albicans icl1 Δ /icl1 Δ* mutant cannot grow on lactate, suggesting that the pyruvate decarboxylase pathway is the preferred route under these conditions and implying a key role for the glyoxylate shunt to synthesize C_4 carbon compounds for gluconeogenesis.

Several studies have now shown that following phagocytosis by macrophages or neutrophils, *C. albicans* cells downregulate glycolytic genes and activate glyoxylate cycle and gluconeogenic genes, allowing the assimilation of two-carbon compounds (1, 8, 15, 16, 21). The role of the glyoxylate cycle and gluconeogenesis in *C. albicans* pathogenesis has been substantiated by analysis of deletion mutants lacking the key enzymes of these metabolic pathways (1, 16; this study). Our data indicate that peroxisomal fatty acid β -oxidation, despite the strong induction of the enzymes of this pathway after phagocytosis by macrophages, does not significantly contribute to the ability of *C. albicans* to survive in vivo. Furthermore, since the glyoxylate cycle was shown to be essential for fungal virulence, our data suggest that, in vivo, the acetyl-CoA required to feed the glyoxylate cycle is derived not from the breakdown of fatty acids but from other nonfermentable carbon sources such as acetate or lactate.

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