A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption

Raimund Eck, Susanne Hundt, Albert Härtl, Ernst Roemer and Waldemar Künkel

Author for correspondence: Raimund Eck. Tel: +49 3641 656852. Fax: +49 3641 656652. e-mail: reck@pmail.hki-jena.de

Hans-Knöll-Institut für Naturstoff-Forschung eV, Abteilung Mikrobielle Infektionsbiologie, Beutenbergstrasse 11, D-07745 Jena, Germany

INTRODUCTION

Most organisms require iron as a cofactor for the catalytic subunits of many proteins. In human tissues only a low level of available iron exists, because iron is sequestered in cells or complexed with high-affinity iron-binding proteins such as ferritin and transferrin. This is used as a general defence mechanism against invading pathogens. Therefore, pathogenic organisms must have mechanisms to obtain iron from the host in order to establish an infection.

Pathogens accumulate iron by different mechanisms. The most widespread mechanism involves the excretion of iron chelators (siderophores). Siderophore production occurs in various pathogenic fungi (Holzberg & Artis, 1983). One study reported that the dimorphic human pathogenic yeast *Candida albicans* can secrete siderophores of both the hydroxamate and the phenolate type (Ismail *et al*., 1985). Another report only confirmed the production of the hydroxamate type (Sweet & Douglas, 1991). However, it is not clear how *C. albicans* obtains Fe(II) from the ferric-siderophore complex or from other sources. Little is known about siderophore-independent mechanisms of iron uptake of *C. albicans*, although Morrissey *et al.* (1996) determined a ferric reductase activity regulated similarly to the *S. cerevisiae* ferric reductase, which was induced in low-iron medium.

The well-characterized yeast *Saccharomyces cerevisiae* does not secrete its own siderophores. It uses foreign siderophores and a two-step reductive Fe(III) uptake system. The two steps are the reduction of Fe(III) to Fe(II) by the surface ferric reductases Fre1p and Fre2p (Dancis *et al*., 1994; Georgatsou & Alexandraki, 1994), and the transport (and oxidation?) by a low- or high-affinity Fe(II) transport system (Dix *et al*., 1997; Askwith *et al*., 1994). The high-affinity system has a $K_m$ for iron of 0·15 µM (Eide *et al*., 1992). The low-affinity system works at iron concentrations higher than 5 µM. The yeast Fet4p is responsible for the low-affinity iron-transport system. The yeast Fet3p is required for high-affinity iron transport and works downstream of the ferric reductases. It encodes a cell-surface ferroxidase with homology to the multicopper oxidase family (De Silva *et al*., 1995). It is a type 1 membrane protein with only one potential transmembrane domain, in which the

**Abbreviation**: BPA, bathophenanthrolinedisulfonic acid.

The EMBL accession number for the sequence reported in this paper is Y09329.
ferroxidase domain is located on the extracellular surface (De Silva et al., 1995). The transcription of FET3 is regulated by the transcription factor Aft1p and the iron concentration. Transcripts for Fre1p, Fre2p and Fet3p are not detected in the absence of a functional AFT1 gene (Yamaguchi et al., 1995). Cells grown in low-iron medium (1–10 μM) contain abundant FET3 mRNA, while cells grown in high-iron medium (1000 μM) show no detectable FET3 mRNA (Askwith et al., 1994). The high-affinity iron uptake system in S. cerevisiae requires copper. Copper deficiency or mutations in genes involved in delivery of copper to Fet3p [CTR1, encoding the cellular copper uptake transporter (Dancis et al., 1994); CCC2, encoding the intracellular copper transporter (Yuan et al., 1993)] abrogate iron uptake as a secondary effect. The human multicopper oxidase ceruloplasmin exhibits similarity to the yeast Fet3p oxidase. Ceruloplasmin plays an important role in iron uptake by human cells.

The amino acid sequence of Fet3p shows no homology to the family of permeases and it has only a single transmembrane domain. Therefore, it is difficult to understand how Fet3p could mediate the iron transport. A candidate for the transport protein is Ftr1p. This protein contains six transmembrane domains and shows homology to Fth1p, a permease of unknown function (Stearman et al., 1993).

In a screen of genes which suppress the iron-limited lack of growth of fet3 fet4 mutants, Spizzo et al. (1997) isolated another multicopper oxidase from S. cerevisiae (Fet5p). Fet5p shows an identity of 47% to Fet3p and the same structural features. It has the same function as Fet3p in iron transport. However, the fet5 mutant is able to grow in iron-limited medium, in contrast to the fet3 mutant (Spizzo et al., 1997). Two potential roles for Fet5p in iron metabolism have been proposed: FET5 may encode a Fet3p isoenzyme, or Fet5p may be involved in transport of iron across the membrane of an intracellular compartment such as the vacuole.

In this study we cloned a gene encoding a multicopper oxidase, Fet3p, in C. albicans and investigated whether Fet3p is essential for high-affinity iron uptake in vitro. We also examined its role in the pathogenicity of C. albicans.

METHODS

Strains and culture conditions. Escherichia coli XL-1 Blue (supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F’ [proAB lacI M15 Tn10(Tet)]) (Stratagene) was used for cloning experiments. C. albicans BMSY 212, a clinical isolate, was used for PCR. C. albicans SC5314 was used as wild-type strain in the growth analysis and in pathogenicity tests (Fonzi & Irvine, 1993). C. albicans 1161 was used for the preparation of a library in a fosmid vector (constructed by M. Strathmann, Stanford University, CA, USA). C. albicans CAI-4 (URA3::imm434/URA3::imm434), derived from the wild-type strain SC5314, was used as the parental strain for the CaFET3 gene disruption. C. albicans was routinely cultured in YPD medium [2% (w/v) d-glucose, 2% (w/v) peptone, 1% (w/v) yeast extract] at 28 °C. C. albicans and S. cerevisiae transformants were spread on YNB plates (0.7% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) Noble agar).

Comparative studies of the growth of the homozygous fet3Δ mutant strain and the wild-type parental strain SC5314 were carried out as follows. Various concentrations of iron (FeCl3, 0–3 μM), equal numbers of cells from the fourth generation in LIM medium (containing approximately 0.2 μM iron as estimated by atom mass spectroscopy) (Eide & Guarente, 1992) and 300 μM bathophenanthrolinedisulfonic acid (BPA; Sigma-Aldrich) were added to LIM medium supplemented with 300 μM BPA. In addition, similar growth experiments were performed in YNB medium supplemented with various iron concentrations (0–3 μM), 180 μM dipiridyl (Sigma-Aldrich) and Candida cells from a YNB culture [180 μM dipiridyl, 10 μM iron]. BPA only binds extracellular iron; dipiridyl chelates extra- and intracellular iron. S. cerevisiae DY1457 (MATα ade6 can1 his3 len2 trp1 ura3) and S. cerevisiae DEY1394 (MATα ade6 can1 his3 len2 trp1 ura3::FET3-2::HIS3) were used for the complementation experiments (Askwith et al., 1994). These strains were grown in YNB supplemented with 40 or 80 μM BPA. For iron-deficiency growth experiments, only plastic bottles were used.

PCR. The low-stringency PCR amplification reactions were carried out in a 100 μl reaction volume containing 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dNTP and 0.2 μM of each primer. The reaction was started by addition of 1 unit Taq polymerase (Boehringer Mannheim) and the samples were overlaid with mineral oil. Thirty cycles were performed consisting of 30 s incubation at 96 °C, 1 min at 42 °C and 2 min at 72 °C using an Omn-E (Hybaid) thermal cycler. Degenerate oligonucleotide primers REBA (5’TAKCAKATHTTYGAR-3’) and REBEI (5’TCTYGNGARCTYTCNGT-3’) were derived using the REBI consensus sequences of S. cerevisiae REBI (dRNA enhancer binding protein) and Kluyveromyces lactis REBI (Morrow et al., 1993). The sequences of the primers were adapted to the codon usage in C. albicans (Lloyd & Sharp, 1992) because the DNA of C. albicans is A/T-rich (the third position of triplets mostly contains A or T). The primers show insignificant homology to CaFET3. RT-PCR was performed by standard procedures (Ausubel et al., 1995).

Cloning and sequence analysis of CaFET3. The CaFET3 PCR probe was used to screen a fosmid library. A 70 kb PstI probe-reactive fragment was subcloned (pFOS1FET3). A 3.3 kb HindIII fragment of the insert DNA of pFOS1FET3 was cloned into pUC19, yielding plasmid pH3300, and sequenced (Fig. 1). The nucleotide sequences were determined by the dyeode chain-termination method with synthetic oligonucleotide primers using the Sequenase version 2.0 kit (United States Biochemical). All other recombinant DNA procedures were carried out by standard protocols (Sambrook et al., 1989). Alignments were performed using the GCG software package (Genetics Computer Group). Block searches were performed by the method of Henikoff & Henikoff (1994) (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Homology searches were performed using the BLAST computer programs (Altschul et al., 1990).

Construction of plasmids pDV1 and pDV2. We amplified the 5′ region of CaFET3 by PCR using chromosomal DNA of C. albicans SC5314 as a template and primers 1 and 2 [primer 1 (5′-GCGGAGCTCGGTTGAACATAACTTGTACACTTC-3′), underlined sequence is complementary to the sequence of
CaFET3, nucleotide position +319 to +342; +1 is the first base of the ATG translation start codon; primer 2 (5'-GGCGTACCTGCTTCTTGATTTAAATCTGG-3', +553 to +530). The resulting 335 bp fragment was digested with SacI/KpnI and cloned in front of the hisG-URA3-hisG cassette (+1 kb) of pMB-7 (Fonzi & Irvine, 1993) to give pDV1. Primer 3 (5'-GAAGCTTATCGAGATCC- TCAAGCCTTCAA-3', +1489 to +1512) and primer 4 (5'-GCTTCAGAAGCTTCCTCCGTATCAT- 3', +1825 to +1802) were used to amplify the 3' end of CaFET3. The resulting 337 bp fragment was digested with HindIII/PstI and cloned into pDV1. The resulting plasmid pDV2 containing the hisG-URA3-hisG cassette flanked by the 5' and 3' regions of CaFET3 was used for gene disruption (Fig. 1).

Transformation of C. albicans, isolation of heterozygous and homozygous multicopper oxidase mutants and selection of Ura– auxotrophs. The methods were carried out by procedures described previously (Boeke et al., 1984; Swoboda et al., 1995; Eck et al., 1997).

Southern blot analysis and transformation of S. cerevisiae DEY1394 fet3A. Southern blot analysis was performed by standard procedures (Sambrook et al., 1989). Transformation was performed according to Klebe et al. (1983).

Preparation of total RNA from C. albicans SC5314 and S. cerevisiae DY1457. Because the S. cerevisiae Fet3 gene was expressed in cells grown in low-iron LIM medium, CaFET3 mRNA was also isolated from cells grown in low-iron medium. Late-exponential-phase C. albicans SC5314 cells from the fourth passage (LIM medium, 300 µM BPA) were harvested by centrifugation and resuspended in two different media: 1, 300 ml LIM medium was supplemented with 300 µM BPA and 0.1, 1, 10 or 100 µM iron; 2, 300 ml YNB medium supplemented with 180 µM bipyridyl and 0.1, 1, 10 or 100 µM iron. S. cerevisiae DY1457 cells from a late-exponential-phase YNB culture were harvested and resuspended in 300 ml LIM medium supplemented with 1 µM iron. After different periods of growth (C. albicans, 10 min to 24 h; S. cerevisiae, 3 h) the cells were harvested and resuspended in 10 ml 50 mM sodium acetate, 10 mM EDTA, pH 5.3, 10 ml phenol (Aqua-Rotiphom; Roth) 100 µl 10% (v/v) SDS and incubated twice for 5 min at 65 °C followed by 5 min in liquid nitrogen. After centrifugation, the aqueous phase was extracted with phenol and with phenol/chloroform (1:1, v/v). After ethanol precipitation the RNA was dissolved in 80% (v/v) ethanol and quantified on the basis of A260.

Northern blot analysis. Northern analysis was performed using the internal 1-65 kb EcoRI–MunI fragment of CaFET3 (+195 to +1836) and an internal PCR product of S. cerevisiae Fet3 (+377 to +1600) as probes. RNA samples (20 µg) were separated in formaldehyde gels containing 1% (w/v) agarose. The RNA was blotted onto Hybond nylon membranes (Amersham). Membranes were hybridized and washed by standard techniques (Sambrook et al., 1989).

Characterization of the mutant strains. The C. albicans SC5314 wild-type strain and the C. albicans fet3A mutant (fet3A::hisG::fet3A::hisG-URA3-hisG) were grown at 28 °C in 10 ml LIM medium with 300 µM BPA over five passages each of 5 d. The fifth passage did not grow. One hundred microlitres of cell suspension from the fourth passage was transferred into fresh LIM medium with different concentrations of iron (0, 0.01, 0.03, 0.05, 0.1, 0.3, 1 or 3 µM) and 300 µM BPA. The OD600 was measured after 48 h and 72 h. In further experiments both strains were cultured in YNB supplemented with 180 µM bipyridyl and 0, 0.1, 1 and 3 µM iron.

Pathogenicity assays. We used the mouse candidiasis model described by Plempel (1984) to test C. albicans mutants for pathogenicity. Briefly, wild-type or mutant strains were grown for 24 h at 28 °C in YNB medium. Ten mice (eight week old, male, NMRI mice, Halan-Winkelmann, Paderborn, Germany) were infected by injection into the caudal vein of 5 × 106 or 5 × 105 cells in 0·2 ml PBS. Survival was monitored for 14 d.

Adherence assay. Mouse L929 fibroblasts were kindly provided by H.-M. Dahse from our institute. The adherence fluorescence assay was carried out by the protocol of Borg-von Zepelin & Wagner (1995). Briefly, the fibroblasts were incubated in microtest plates; C. albicans cells (2 × 106 cells ml–1), preincubated and stained with calcofluor white, were then added, followed by incubation for 2 h at 37 °C. Calcofluor white binds predominantly to chitin and glucan in the fungal cell wall. Non-adherent Candida cells were removed. Finally, the amount of adherent fluorescent C. albicans cells was determined by an automatic fluorescence reader (FluoroScan; Labsystems) (absorption, 360 nm; emission, 460 nm).

RESULTS

Cloning of a C. albicans gene encoding a multicopper oxidase

Five PCR products amplified from chromosomal DNA of C. albicans BMSY 212 with the degenerate primers REBA and REBE1 were cloned into the pCR-Script Amp

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**Fig. 1.** Restriction maps of the plasmids pH3300 and pDV2. (a) The CaFET3 gene (arrow, coding region; thick black boxes, non-coding regions) was cloned into pUC18, yielding plasmid pH3300. (b) The 5' and 3' regions of CaFET3 obtained by PCR were cloned in front of or behind the hisG-URA3-hisG cassette (4.1 kb) in the disruption vector pMB-7. Horizontal black arrows (P1, P2, P3, P4) indicate the location of sequences to which the primers were complementary. Vertical black arrows show the location of restriction sites as follows: E, EcoRI; H, HindIII; K, KpnI; M, MunI; P, PstI; Sa, SacI; S, SalI; Sc, Scal; S, Smal; X, XbaI.
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Fig. 2. Multiple alignment for maximal amino acid similarities of multicopper oxidases from C. albicans (cafet3), S. cerevisiae (scfet3, scfet5) and Sch. pombe (schpfet). Similar residues are indicated by white letters in black boxes.

SK(+) cloning vector. Sequence analysis revealed only one amplimer with homology to a known sequence. It showed 65% identity at the amino acid level with the multicopper oxidase Fet3p of S. cerevisiae. This insert was used as a probe to screen a C. albicans 1161 fosmid library. Two probe-reactive clones were isolated (termed 2/C6 and 14/A10). The data evaluation of the fosmid-library screening database showed that the inserts of both clones had been previously localized on chromosome 6, indicating that the C. albicans FET3 homologue (CaFET3) was localized on chromosome 6.

The two fosmids were digested with PstI. Southern analysis of one fosmid showed a 7–0 kb band that hybridized with the PCR probe. This fragment was subcloned into plasmid pUC18, yielding the plasmid pFOS1FET3. After digestion of pFOS1FET3 with HindIII and subsequent hybridization with the PCR probe, a 3.3 kb probe-reactive fragment was identified and cloned into pUC18, yielding plasmid pH3300. This fragment was subsequently found to contain the entire CaFET3 gene (Fig. 1).

Sequence analysis and characterization of CaFET3

The 3.3 kb insert of pH3300 contained one ORF of 1872 bp. This ORF encoded a hypothetical polypeptide of 624 amino acids with a predicted molecular mass of 70.5 kDa. The isoelectric point was predicted to be at a pH value of 4.63. Two TATA boxes at nucleotide positions −113 to −117 and −129 to −135 and a CAAT box at position −142 to −145 were located. In amino acid sequence comparisons, the product of the identified ORF from C. albicans showed the highest overall identity to the multicopper oxidase Fet3p from S. cerevisiae (55%). The ORF was therefore named CaFET3. Identities were also found between the putative CaFet3p and the multicopper oxidases Fet5p from S. cerevisiae (52%) and Fet3p from Schizosaccharomyces pombe (40%) (Fig. 2).

Block searches revealed four amino acid sequences that correspond to copper-binding sequences of the family of multicopper oxidases [amino acid (aa) positions 121 to 142, 333 to 350, 412 to 420 and 476 to 487] (Fig. 2). Members of this family are laccases, ceruloplasmin and...
ascorbate oxidase, which mediate the reduction of oxygen to water by a four-electron transfer. We found homologies between CaFet3p and each of the members of the multicopper oxidase group. Strong homologies were shown between CaFet3p and different laccases; the highest identities between CaFet3p and different laccases exist around the copper-binding sequences (white-rot basidiomycete Coriolus hirsutum Lac1p, aa 413 to 421, 100% identity; aa 77 to 139, 56% identity; aa 469 to 499, 78% identity). The human homologue to ScFet3p, ceruloplasmin, is also a multicopper oxidase and shows sequence homology to CaFet3p around three potential copper-binding regions (aa 104 to 121, 44% identity; aa 413 to 423, 64%; aa 456 to 492, 32%).

The hydrophobicity plots (Kyte–Doolittle) of Fet3p and CaFet3p showed potential transmembrane domains. These were identified in the C-terminal region of the proteins (Fet3p and CaFet3p: aa 1 to 21). These are potential secretory signal sequences (von Heijne, 1983). The putative transmembrane domains and the potential secretory signal sequences showed similarities of 70% and 45%, respectively (De Silva et al., 1995).

Southern blot analysis was performed applying the same hybridization and washing conditions as used for the fosmid screening, and using the PCR product as a probe. The results implied that CaFET3 is present as a single copy per haploid genome in C. albicans SC5314 and that no other homologous genes occur in the C. albicans genome.

CaFET3-specific mRNA was not detected by Northern blot analysis of total RNA of C. albicans SC5314. Experiments with different time periods of growth (10 min to 24 h), concentrations of iron (0·1 to 100 µM) and media (LIM medium, YNB, YED) and with the iron chelators BPA and dipyridyl, did not lead to a detectable mRNA. According to expectations, the control Northern blot analysis of S. cerevisiae DY1457 RNA showed an approximately 2·1 kb hybridizing FET3 mRNA. To detect CaFET3 mRNA, RT-PCR was performed. We used primers 1 and 2 (see Methods, construction of pDV1) and total RNA from C. albicans SC5314 grown in LIM medium supplemented with 0·1, 1, 10 or 100 µM iron. We detected a 235 bp product in each RT-PCR experiment. This result indicated that the CaFET3 was expressed under both low- and high-iron conditions, although at low abundance.

**Disruption of CaFET3**

*C. albicans* CAI-4 was transformed with the *hisG-URA3-hisG* cassette flanked by S′ and S′ CaFET3 gene sequences obtained by digestion of pDV1 with *SalI*/*HindIII* (Fig. 1). DNA from a representative Ura+ colony was isolated and digested with *PstI*. Southern analysis with the *32P*-labelled 3·3 kb *HindIII* insert of plasmid pH3300 (Fig. 1) showed two hybridizing fragments (Fig. 3, lane 2). One band is identical to the 7·0 kb hybridizing fragment of the parental strain CAI-4 shown in lane 1. The length of the second band of 10·1 kb is consistent with the replacement of 50·5% of the CaFET3 gene by the *hisG-URA3-hisG* cassette.

The disruption of the second CaFET3 allele required as a first step the loss of the selectable marker gene *URA3*. The selection of Ura− segregants on 5-fluoro-orotic acid plates gave approximately 10⁸ *C. albicans* CAI-4 *FET3/fet3Δ::hisG Ura−* colonies. Southern analysis of DNA from a representative colony showed two hybridizing fragments (Fig. 3, lane 3). One band is identical to the 7·0 kb band of the parental strain shown in lane 1. The length of the second hybridizing fragment of 7·2 kb is consistent with the loss of the *URA3* gene and one copy of *hisG* from the *hisG-URA3-hisG* cassette.

The gene replacement was repeated to disrupt the second allele of CaFET3 gene in *C. albicans*. Five putative homozygous clones (*fet3Δ::hisG/fet3Δ::hisG-URA3-hisG*) were obtained. Southern analysis of chromosomal DNA from a representative clone is shown in Fig. 3, lane 4. One hybridizing fragment is identical to the 7·2 kb band of *C. albicans* CAI-4 *FET3/fet3Δ::hisG* shown in lane 3. The second hybridizing fragment is identical to the 10·1 kb band of *C. albicans* CAI-4 *FET3/fet3Δ::hisG-URA3-hisG* shown in lane 2. The 7·0 kb fragment of the parental strain CAI-4 was missing.

**Characterization of the fet3Δ mutant**

The wild-type strain *C. albicans* SC5314 and the mutant derivative *C. albicans* fet3Δ (*fet3Δ::hisG/fet3Δ::hisG-URA3-hisG*) showed different growth in iron-limited medium (0·01–3 µM iron) (Fig. 4). The growth of *C. albicans* SC5314 and *C. albicans* fet3Δ was inhibited without addition of iron to the LIM medium supplemented with 300 µM BPA in the fifth passage. The addition of 0·01–0·3 µM iron facilitated the growth of *C.
albicans SC5314 but not that of *C. albicans fet3Δ*. The mutant strain grew after addition of at least 1.0 µM iron and an incubation period of 48 h. The chelator bipyridyl (180 µM in YNB medium), which bound extra- and intracellular iron, caused a dramatic decrease of growth in iron-limited medium. However, the results after addition of iron to YNB medium supplemented with bipyridyl were similar to those for BPA in LIM medium (data not shown). Together with the data from the comparison between Fet3p and Fet5p of *S. cerevisiae* and CaFet3p from *C. albicans*, it can be concluded that we have isolated a *C. albicans* multicopper oxidase gene involved in high-affinity iron uptake.

We investigated the pathogenicity of the wild-type strain *C. albicans* SC5314 and the mutant derivative *C. albicans fet3Δ* in the mouse model of systemic candidiasis. We found only insignificant differences in pathogenicity between these two strains, at both the infection dosages tested (5 × 10⁶ and 5 × 10⁷ cells) (Fig. 5).

We tested the adhesion of *C. albicans fet3Δ* mutant cells to mouse L929 fibroblasts. Three independent tests showed that the adhesion of the mutant strain *C. albicans fet3Δ* to mouse L929 fibroblasts was reduced to 56% compared with the adhesion of the wild-type strain *C. albicans* SC5314 (100%). The standard deviation was ±3%.

Complementation of *S. cerevisiae DEY1394 fet3Δ* with CaFET3

Due to the role of Fet3p in high-affinity iron uptake, the knockout mutant strain *S. cerevisiae DEY1394 fet3Δ* is unable to grow on iron-limited media, either complex medium with 80 µM of the iron chelator BPA or LIM medium (Askwith *et al.*, 1994). This observation was the basis for our complementation experiments. We transformed *S. cerevisiae DEY1394 fet3Δ* with YCp50CaFET3 containing the 3.3 kb *HindIII* fragment of pH3300. Cells were plated on YNB plates without uridine so that only transformants bearing a plasmid were able to grow. Plasmid-rescue experiments were performed from three transformants. All three transformants contained YCp50CaFET3. One transformant (*S. cerevisiae DEY1394 fet3Δ* containing YCp50CaFET3), the mutant strain *S. cerevisiae DEY1394 fet3Δ* and the wild-type strain *S. cerevisiae DY1457* were grown in YED medium with 40 µM and 80 µM BPA, respectively. If the medium contained 80 µM BPA only *S. cerevisiae DY1457* grew. This result showed that at this concentration of BPA the CaFET3 gene was unable to restore the growth of *S. cerevisiae DEY1394 fet3Δ* in iron-limited medium. However, CaFET3 was able to complement the growth deficiency of *S. cerevisiae DEY1394 fet3Δ* in iron-limited medium with 40 µM BPA (Fig. 6).
DISCUSSION

The FET3 gene of \textit{C. albicans}, \textit{CaFET3}, was isolated and sequenced. Sequence analysis of the gene revealed an ORF of 624 amino acids with the highest homology to the iron-transport multicopper oxidase Fet3p from \textit{S. cerevisiae} (55\% identity). The ORF also showed significant homology to Fet5p (52\% identity), a second multicopper oxidase from \textit{S. cerevisiae} involved in iron uptake (Spizzo et al., 1997). This homology is higher than that between Fet3p and Fet5p from \textit{S. cerevisiae} (47\% identity). Nevertheless, we assume that the cloned gene is homologous to \textit{S. cerevisiae} FET3 because \textit{S. cerevisiae} \textit{fet5A} is able to grow in iron-limited medium in contrast to \textit{S. cerevisiae} \textit{fet3A} and \textit{C. albicans} \textit{fet3A}. Further, the \textit{FET5}- and \textit{FET3}-dependent uptake of iron in \textit{S. cerevisiae} had an apparent \( K_m \) of 5 \( \mu \)M and 0.5 \( \mu \)M, respectively. We did not determine the exact \( K_m \) of \textit{CaFET3}-dependent uptake, but the growth of \textit{C. albicans} \textit{fet3A} in iron-limited medium (0–3 \( \mu \)M iron) indicated a \( K_m \) of approximately 0.5 \( \mu \)M. This value is in good agreement with the \( K_m \) of \textit{FET3}-dependent uptake of iron in \textit{S. cerevisiae} (Eide et al., 1992).

The fact that wild-type \textit{C. albicans} needed five passages before growth was inhibited in LIM medium (with approximately 0.2 \( \mu \)M iron) containing the extracellular chelator BPA indicated that a low-affinity iron uptake mechanism exists in \textit{C. albicans}. We assume that siderophores and the intracellular pool of iron contribute to the long survival of \textit{C. albicans} in the iron-limited medium. Moreover, it is possible that our strain is unable to produce siderophores. Differences in siderophore production between \textit{C. albicans} strains have been observed (Ismail et al., 1985; Sweet & Douglas, 1991).

Unlike \textit{C. albicans} \textit{fet3A}, \textit{S. cerevisiae} \textit{fet3A} showed growth inhibition in YPD medium containing 40 \( \mu \)M BPA, because it produces none of its own siderophores or/and it possesses no effective mechanism of iron storage.

We did not succeed in detecting \textit{CaFET3} mRNA following Northern analysis with a \textit{FET3}-specific probe. However, RT-PCR experiments showed that \textit{CaFET3} mRNA was produced at low abundance. There are three possible reasons for these results: firstly, a fast turnover of \textit{CaFET3} mRNA; secondly, a low level transcription of \textit{CaFET3}; and thirdly, unusual conditions for the initiation of transcription compared with \textit{S. cerevisiae} FET3.

Comparative analysis of the pathogenicity of the wild-type strain \textit{C. albicans} SC5314 and the mutant derivative \textit{C. albicans} \textit{fet3A} showed no differences in virulence, although the ability of pathogens to accumulate iron is considered as a virulence factor. Possible reasons for our results include the following: the storage of iron in the inoculated \textit{Candida} cells may have been too high (first culture in LIM medium with 300 \( \mu \)M BPA); the mutant strain may have been able to produce enough siderophores; or the mutant may have used other means of iron uptake.

Fratti et al. (1998) found that chelation of endothelial cellular iron protected these cells from injury by \textit{C. albicans} and that adherence was slightly enhanced. We also found a correlation between iron metabolism and adherence of \textit{C. albicans} to mouse fibroblasts. Possibly the decreased adherence of \textit{C. albicans} \textit{fet3A} was mediated by modified production of reactive oxygen intermediates caused by an incomplete reduction of oxygen.

Two essential proteins of the high-affinity iron uptake system have now been identified in \textit{C. albicans}. Morrissey et al. (1996) determined a cell-associated ferric reductase activity in \textit{C. albicans} showing identical features to those of the homologue \textit{S. cerevisiae} enzyme. Additionally, our results have shown that \textit{C. albicans} \textit{FET3} encodes a homologous protein to the \textit{S. cerevisiae} multicopper oxidase Fet3p which is responsible for the high-affinity transport of Fe(II). This study demonstrates for the first time the functional similarity between the high-affinity iron uptake systems of \textit{S. cerevisiae} and \textit{C. albicans}. Therefore, it seems that the basic elements of the high-affinity iron uptake system of the pathogenic yeast \textit{C. albicans} and the non-pathogenic yeast \textit{S. cerevisiae} are identical.

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