Reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) is dependent on *CaFRE10* ferric reductase for *Candida albicans* grown in unbuffered media

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The reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) and other tetrazolium salts is widely used as an assay for bacterial, fungal and mammalian cell viability, but the genes encoding the reductase activities have not been defined. Here, it was shown that XTT and plasma membrane ferric reductase activities were 10–40-fold greater in *Candida albicans* than in *Saccharomyces cerevisiae*. XTT reductase activity was induced fivefold in *C. albicans* grown in low-iron conditions compared with iron-replete conditions, and for cells grown in unbuffered (pH 4.0–4.4) medium, XTT reductase activity was largely dependent on *CaFRE10*. XTT reductase activity of *C. albicans* grown in medium buffered to pH 6.8 was independent of *CaFRE10* but, nonetheless, was upregulated in cells deprived of iron. Reduction of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT), a membrane-permeable tetrazolium salt, occurred at an intracellular location and was independent of *CaFRE10*. However, MTT activity was induced by iron deprivation in *C. albicans* but not in *S. cerevisiae*. *C. albicans* possessed multiple iron- and pH-regulated reductase activities capable of reducing tetrazolium salts, but, when grown in unbuffered medium, *CaFRE10* was required for XTT reductase activity.

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INTRODUCTION

Tetrazolium salts are widely used as indicators of cellular proliferation and biomass for eukaryotic and prokaryotic cells (Johnsen *et al.*, 2002; McCluskey *et al.*, 2005; Roehm *et al.*, 1991; Scudiero *et al.*, 1988). Live cells reduce the tetrazole ring and a coloured formazan product is formed, which can be assessed visually and quantified spectrophotometrically. Such assays are used to test the efficiency of antifungal drugs in killing or inhibiting growth of *Candida albicans* and other fungi (Heyn *et al.*, 2005; Kuhn *et al.*, 2003; Meletiadis *et al.*, 2001; Tellier *et al.*, 1992; Wiederhold *et al.*, 2005). The tetrazolium salts used in biology are aromatic derivatives of 1,2,3,4-tetrazole and include monotetrazolium [e.g. 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), 2-(4,5-dimethyl-2-thiazolyl)-

3,5-diphenyl-2H-tetrazolium bromide (MTT) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1)] or ditetrazolium [e.g. nitro blue tetrazolium (NBT)] variants (reviewed by Berridge *et al.*, 2005). XTT and WST-1 are reduced to form soluble coloured formazan products (Ishiyama *et al.*, 1993; Paull *et al.*, 1988; Peskin & Winterbourn, 2000), and MTT, which can cross cellular membranes, forms water-insoluble formazan crystals upon reduction (Berridge *et al.*, 2005; Mosmann, 1983).

Despite widespread use of these assays, the gene(s) and encoded enzymes that catalyse the reduction of the tetrazolium salts are still not known. Reduced pyridine nucleotides are likely to be the originating electron donors, but the specific reductases involved remain to be identified (Berridge & Tan, 1993; Vistica *et al.*, 1991). In mammalian cells, the plasma membrane is thought to be the site for the extracellular reduction of the soluble tetrazolium salts (e.g. XTT and WST-1), in a process that might involve NADH coenzyme Q-dependent ferricyanide reductases and a ubiquinone-dependent plasma membrane electron transport chain (Berridge & Tan, 2000; Crane *et al.*, 1985; Kim *et al.*, 2002; Morre, 2004; Villalba *et al.*, 1995).

Abbreviations: 1-mPMS, 1-methoxy-5-methylphenazinium, methyl-sulfate; BPS, bathophenanthroline disulfonate; FRE, ferric reductase; mtDNA, mitochondrial DNA; MTT, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; NOX, NAD(P)H oxidase; SOD, superoxide dismutase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt.

Whole cells of *Saccharomyces cerevisiae* can also reduce ferricyanide (Crane *et al.*, 1982), and externally directed ferric reductases (FREs), encoded by the genes *ScFRE1* and *ScFRE2*, are responsible for this process (Dancis *et al.*, 1992; Georgatsou & Alexandraki, 1994). The FREs have wide phylogenetic distribution (Lara-Ortiz *et al.*, 2003), and, in *C. albicans*, there are at least 11 FRE family members (Arnaud *et al.*, 2005; <http://www.candidagenome.org/>). The FREs are, in turn, related to the large NAD(P)H oxidase (NOX) protein family. The archetypical NOX is gp91phox, which is present in human phagocytes, and responsible for superoxide production within the phagocytic vacuole (reviewed by Segal, 2005). Key features of NOX proteins that are conserved in FRE proteins include four conserved histidine residues for coordination of two *b*-haem groups within an intramembraneous region, and amino acid motifs associated with FAD and NADPH binding (Finegold *et al.*, 1996). This configuration of cofactors permits the energetically favourable transfer of intracellular electrons from intracellular NADPH across the plasma membrane to an extracellular electron acceptor (Vignais, 2002).

In *S. cerevisiae* and *C. albicans*, a primary biological role of the FREs is the reduction of ferric iron chelates, thereby releasing ferrous iron and making it available to the high-affinity iron permease system, which consists of a multicopper oxidase (FET) and an iron permease (FTR) (Askwith *et al.*, 1994; Eck *et al.*, 1999; Knight *et al.*, 2002; Kosman, 2003; Ramanan & Wang, 2000; Stearman *et al.*, 1996). This cellular iron-uptake system is critical for growth in iron-limiting conditions, and is homeostatically upregulated at the level of transcription in response to low levels of environmental iron (Blaiseau *et al.*, 2001; Braun *et al.*, 2000; Courel *et al.*, 2005; Knight *et al.*, 2002; Lan *et al.*, 2004; Rutherford *et al.*, 2005; Yamaguchi-Iwai *et al.*, 1996). In studying the reductive iron-uptake system, we observed that *C. albicans* was able to reduce ferric and take up ferrous iron at a 40–100-fold greater rate than that of *S. cerevisiae* (Knight *et al.*, 2005). *ScFre1p* is known to have broad substrate specificity, and can utilize a variety of electron acceptors as substrates, including nitroprusside, cupric chelates, resazurin and azo dyes, as well as ferric chelates (Hassett & Kosman, 1995; Lesuisse & Labbe, 1994; Ramalho *et al.*, 2005). This led us to investigate if *CaFre10p*, the predominant externally directed ferric reductase of *C. albicans* when grown in typical laboratory conditions (Knight *et al.*, 2005), was responsible for the reduction of tetrazolium salts used in the viability/biomass assays of *C. albicans*.

METHODS

Strains. *C. albicans* SC5314 was used as the wild-type reference strain. Other wild-type strains examined were CBS5736 (ATCC 32032) and a high-frequency switching strain, WO-1, kindly provided by David Soll, University of Iowa (Slutsky *et al.*, 1987). Strain BCal5-2 harbours a homozygous deletion of *CaFRE10* (also known as *RBT2*, *CFL95*) (*CaFre10Δ::hisG/fre10::hisG-URA3-hisG ura3Δ::imm434/ura3Δ::imm434*) and was a gift from Burk Braun and Alexander Johnson, University of California, San

Francisco (Braun *et al.*, 2000). Strain CT218.2 (*CaFre10Δ::hisG/CaFre10::hisG::CaFRE10-URA3 ura3Δ::imm434/ura3Δ::imm434*) harbours a reinserted functional *CaFRE10* and CT212.2 (*CaFre10Δ::hisG/CaFre10::hisG::CaFre10-1-URA3 ura3Δ::imm434/ura3Δ::imm434*) harbours a reinserted mutated *CaFre10* allele, and is auxotrophically matched to CT218.2 (Knight *et al.*, 2005).

S. cerevisiae strains used were YPH499 [*MATA ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 leu2-Δ1*; Sikorski & Boeke, 1991] and the congenic ferric reductase knock-out 499Δ1Δ2 [*MATA ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 Δfre1::LEU2 Δfre2::HIS3*; Finegold *et al.*, 1996]. Strain YPH250 rho⁻ is congenic with YPH499 (except for the presence of a *trp1-Δ1* allele) and was treated with ethidium bromide to ablate mitochondrial DNA (mtDNA) (Fox *et al.*, 1991).

Growth conditions and media. For all experiments, strains were streaked from -80°C glycerol stocks onto 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose and 0.01% (w/v) adenine (YAPD) agar medium, grown for 2 days at 30°C , then transferred to room temperature where they were maintained for a maximum of 10 days. All experiments were initiated from inoculation of a single colony to a single culture. For biological replicates, multiple single colonies were individually inoculated into multiple cultures. Defined iron-deficient medium was prepared from complete supplement mixture (CSM, 0.79 g l^{-1} ; Q-BioGene) supplemented with 0.1 g l^{-1} uridine l^{-1} , yeast nitrogen base (YNB, 6.7 g l^{-1} , USBiologicals) without amino acids, iron, zinc, manganese or copper, and 2% (w/v) D-glucose. This was supplemented with 0.4 mg l^{-1} zinc sulfate l^{-1} , 0.4 mg l^{-1} manganese sulfate l^{-1} and $1\text{ }\mu\text{M}$ copper sulfate. The pH of this unbuffered medium was 4.0–4.4. Milli-Q (Millipore) water was used for all growth media and chemical solutions. All iron-deficient media were prepared in plastic containers and filter-sterilized, and cells were grown in polypropylene or polystyrene vessels. The predicted iron concentration of this low-iron medium was of the order 50–100 nM (Eide *et al.*, 1992).

Ferric, XTT, WST-1, and MTT reductase assays. The protocol for studying the reductase activities could be divided into three distinct stages: stage 1, growth of the cells in the specific medium; stage 2, washing the cells in the assay buffer to remove the growth media; and stage 3, the enzyme assay. Using this protocol, we were able to compare the effects of the growth conditions on the reductase activities under identical enzyme assay conditions.

For all assays, cells were grown in 96-well plates with $150\text{ }\mu\text{l}$ medium at 30°C without shaking. After 17 h, cells were resuspended and subinoculated (1:20) into fresh medium. The freshly inoculated cultures were incubated at 30°C for 5–6 h. Cells were pelleted by centrifugation (1000 g , 5 min) at room temperature and washed twice with standard reductase buffer [50 mM citrate, pH 6.6, with 5% (w/v) D-glucose]. OD_{720} of the cell pellets resuspended in reductase buffer was measured for cell number normalization, and assays were initiated by the addition of appropriate substrate (ferric ammonium sulfate, XTT, WST-1 or MTT; see details below) in reductase buffer to the cell suspensions. Reactions were carried out at 30°C in the dark. After 10–15 min, the ferric, XTT and WST-1 reactions were stopped by the addition of 25% trichloroacetic acid. We have previously determined that the reduction of ferric iron is linear over this time period (Knight *et al.*, 2005). The cells were pelleted by centrifugation (1000 g for 5 min) and the supernatants were transferred to fresh 96-well plates for absorbance measurements (BioTek EL800 plate reader). The MTT reactions were stopped after 1.75 h with acidified (0.04 M HCl) 2-propanol. Cells were pelleted by centrifugation (1000 g , 5 min) and the absorbance of the supernatant was measured. For all assays, reactions containing no cells were used to determine blank values, which were subtracted from values obtained in assays with cells.

Absorbance values of products formed were normalized with optical measurements of culture turbidity. Greater than 99.9 % of cells were in the yeast form.

The ferric reductase assays rely on the formation of a red batho-phenanthroline disulfonate (BPS)–Fe(II) complex, which is quantified by measuring absorbance at 515 nm (Dancis *et al.*, 1990; Lesuisse *et al.*, 1987). The source of ferric iron was 1 mM ammonium ferric sulfate (Fluka) and the concentration of BPS (Sigma) was 1 mM.

The XTT reductase assay relies on the reduction of XTT to form a red formazan product, which is quantified by measuring absorbance at 515 nm. XTT (Sigma) was prepared in standard reductase buffer and used at a concentration of 0.37 mM with 0.5 µM freshly made menadione (Sigma). Menadione was excluded from the XTT assay when superoxide dismutase (SOD) was included, as menadione can spontaneously yield superoxide following enzymic reduction (Hassan & Fridovich, 1979). The absence of menadione decreased overall XTT activity by ~20 %.

The WST-1 assays rely on reduction of WST-1 to form a yellow water-soluble formazan product, which is quantified by measuring absorbance at 450 nm (Ishiyama *et al.*, 1993). WST-1 (Dojindo) was prepared as a 10 mM stock in Milli-Q water and stored as aliquots at –20 °C. Prior to use, an aliquot was thawed, diluted to 1 mM in standard reductase buffer, and added to cells at a final concentration of 0.5 mM. Initial experiments demonstrated that the electron/hydrogen carrier 1-methoxy-5-methylphenazinium methylsulfate (1-mPMS; Dojindo), at 10 µM, had no effect (Student's *t* test, $P=0.845$) on the reduction of WST-1 (data not shown). This recapitulates the lack of effect of 1-mPMS on the reduction of WST-1 by activated neutrophils (Tan & Berridge, 2000). Therefore, 1-mPMS was not included in the WST-1 assays.

The cellular reduction of MTT leads to the formation of insoluble indigo coloured crystals. MTT (Sigma) was prepared as a 1 g l^{–1} stock solution in reductase buffer and combined with cells at a final concentration of 0.5 g l^{–1}. At the end of the assay, the formazan crystals were dissolved in acidified (0.04 M HCl) 2-propanol and the absorbance of the supernatant measured at 595 nm.

In the reactions with iron-containing SOD (Sigma) from *Escherichia coli*, SOD was dissolved in 20 mM HEPES and added at a 1 : 10 dilution to the suspended cells, immediately prior to addition of reductase substrate. Addition of HEPES buffer alone was used as control.

Measurement of oxygen consumption. The rate of cellular oxygen consumption was measured using a Clark-style electrode and a temperature-controlled chamber at 30 °C (System 203; Instech). The analogue signal was converted to a digital signal for data collection and analysis (Powerlab 2/25 and software; ADInstruments). One million cells in exponential phase were diluted into prewarmed fresh media and placed into the sealed chamber. After a 30 s equilibration, recording was started and continued until dissolved oxygen was completely consumed. Oxygen consumption was linear with respect to time during this period.

RESULTS

Ferric reductase and XTT activities are greater in *C. albicans* than in *S. cerevisiae*

NAD(P)H-dependent cell surface reductases are required for iron uptake, the ferrous product entering the cell through the Ftr/Fet-dependent high-affinity system. We previously observed that the rate of ferric iron uptake was

40–100-fold higher in *C. albicans* than in *S. cerevisiae*, and that at least 75 % of this uptake was dependent on CaFRE10 for cells grown in low-iron, unbuffered defined medium (Knight *et al.*, 2005). Direct comparison of whole-cell ferric reductase activity of *C. albicans* (wild-type strain SC5314) to a well-characterized *S. cerevisiae* strain (YPH499) after growth on low-iron defined media, indicated that the cell surface reductase activity of *C. albicans* was at least 10-fold higher than that of *S. cerevisiae* [12.2 ± 2.3 compared to 1.1 ± 0.3 nmol Fe(II) (10⁶ cells)^{–1} h^{–1}, respectively, $n=5$]. Likewise XTT reduction was also considerably greater in *C. albicans* than in *S. cerevisiae* [0.117 ± 0.019 compared to 0.003 ± 0.001 (A₅₁₅)^{–1} (A₇₂₀)^{–1} min^{–1}, respectively; values are means ± SD for $n=5$].

C. albicans XTT surface reductase activities: roles for CaFRE10, iron and pH regulation

The activity of *C. albicans* responsible for the reduction of XTT to the formazan product was regulated by the level of iron in the growth medium prior to the assay (Fig. 1). This iron-regulated activity was reminiscent of the iron-regulated reduction of ferric compounds catalysed by CaFre10p (Knight *et al.*, 2005). To determine if CaFre10p was involved

Fig. 1. *C. albicans* XTT reductase activity is regulated by iron and pH, and is dependent on CaFRE10 in unbuffered medium. *C. albicans* strains SC5314 [wild-type (WT)], BCa15-2 [CaFRE10^{–/–} (Δ)], CT218.2 [CaFRE10^{–/–} + CaFRE10 (R)], and CT212.2 [CaFRE10^{–/–} + CaFRE10-1 (m)] were grown at 30 °C in low-iron defined medium (open bars) or the same medium supplemented with 50 µM ferric ammonium sulfate (solid bars). In exponential phase, the cells were washed and assayed for XTT reductase activity. (a) Results for cells grown in non-buffered medium; (b) results for cells grown in the same medium buffered with 50 mM HEPES to pH 6.8. Data are means ± SD for five independent cultures.

in XTT reduction, a *CaFRE10*^{-/-} homozygous deletion strain (strain BCa15-2; Δ) was compared to SC5314 (WT) (Fig. 1a). When cells were grown in low-iron defined medium and then washed and assayed, wild-type *C. albicans* had 10-fold higher XTT activity than the *CaFRE10*^{-/-} mutant. *C. albicans* CT281.2, harbouring a reinserted functional *CaFRE10* (R), restored XTT activity to 73 % of that of SC5314, whereas reinsertion of a non-functional *CaFRE10-1* allele (strain CT212.2; m) had no effect. This genetic complementation of XTT activity was similar to that observed for ferric reductase activity (Knight *et al.*, 2005). When grown in low-iron conditions, the *CaFRE10*^{-/-} mutant retained some XTT activity. However, when the *CaFRE10*^{-/-} mutant was grown in the presence of iron, the XTT activity was repressed to virtually zero (Fig. 1a). Overall, *C. albicans* XTT activity was regulated by the level of iron exposure during growth. Furthermore, for cells grown in unbuffered medium, in low or iron-supplemented conditions, the majority of the XTT activity was dependent on *CaFRE10*.

The transcript levels of genes *CaFRE1* (orf19.8848), *CaFRE2* (orf19.8849), *CaFRE7* (orf19.6139) and *CaFRE9* (orf19.3538), which have homology to *CaFRE10*, are upregulated at pH 8 (Bensen *et al.*, 2004). We had also previously observed that, for *C. albicans* grown at near-neutral pH, *CaFRE10* contributed very little to the total cell surface ferric reductase activity. In contrast, for cells grown in unbuffered (pH 4.0–4.4) medium, *CaFRE10* encodes the majority of the activity (Knight *et al.*, 2005; and data not shown). To examine how the pH of the growth medium might affect the ability of *C. albicans* to reduce XTT, wild-type and *CaFRE10* mutant strains were grown in defined medium with 50 mM HEPES to maintain pH between 6.7 and 6.8 (Fig. 1b). At this near-neutral pH, XTT activity was independent of *CaFRE10* (Fig. 1b), yet was still regulated by the amount of iron in the growth medium. In addition, the XTT activity of cells grown in buffered medium was two- to threefold greater than for cells grown in unbuffered medium (note different scale of Fig. 1a, b). The identity of the *C. albicans* gene(s) encoding the iron-regulated reductase activity expressed in buffered growth medium is unknown.

Partial inhibition of WST-1 and XTT reduction by SOD

WST-1 is a water-soluble tetrazolium salt that is membrane-impermeable and is reduced to a water-soluble formazan. It can be reduced by superoxide anion, and inhibition of this reaction by SOD can be used to assess superoxide production (Tan & Berridge, 2000). Frequently, with assays that involve reduction of WST-1, an intermediate electron/hydrogen carrier is employed. For WST-1, the electron/hydrogen carrier is 1-mPMS. We observed no effect on the ability of *C. albicans* to reduce WST-1 in the presence or absence of 1-mPMS (data not shown), and therefore did not include 1-mPMS in the assays reported here. To investigate if superoxide was involved in WST-1 and XTT reduction by *C. albicans*, SOD was added to the reactions immediately prior to the addition of WST-1 or XTT (Fig. 2a). In the

presence of 30 $\mu\text{g SOD ml}^{-1}$, WST-1 reductase activity was decreased to 74 % of control values (no SOD). This suggests that the majority of WST-1 reduction was not mediated by superoxide. The presence of SOD (30 $\mu\text{g ml}^{-1}$) had a greater inhibitory effect on XTT reduction, but most XTT reduction (64 % of control activity) was SOD-independent.

The standard assay buffer [50 mM citrate, pH 6.6, 5 % (w/v) D-glucose] that we used to assess WST-1 and XTT reductase activities is identical to the buffer used for the genetic and biochemical characterization of *ScFRE1* in *S. cerevisiae* (Dancis *et al.*, 1990; Lesuisse *et al.*, 1987), and is based on earlier assays used to measure ferricyanide reduction (Avron & Shavit, 1963). Citrate can function as a chemical reductant

Fig. 2. *C. albicans* reduction of soluble tetrazolium salts is partially inhibited by SOD and independent of citrate. (a) Wild-type *C. albicans* was grown in low-iron defined media at 30 °C to exponential phase. The cells were washed and assayed for XTT reductase activity (no menadione) (●) and WST-1 reductase activity (▲) in the presence of SOD. (b) Wild-type (WT) and *CaFRE10*^{-/-} (Δ) *C. albicans* were grown in low-iron defined medium (open bars) and the same medium supplemented with 50 μM ferric ammonium sulfate (solid bars) at 30 °C. At exponential phase, the cells were washed in assay buffers of: 50 mM citrate buffer, pH 6.6, with 5 % (w/v) D-glucose (Std buffer); Dulbecco's PBS, pH 7.2, with 5 % D-glucose (PBSG); and PBSG with 50 mM citrate, pH 7.3 (PBSG+Cit). The cells were assayed for WST-1 reductase activity in the corresponding buffers. Data are means $\pm$ SD for five independent cultures.

and is required for optimal ferric reductase activity of intact yeast cells. To test if citrate might be required to mediate reduction of WST-1, we compared the ability of wild-type and *CaFRE10*^{-/-} *C. albicans* to reduce WST-1 in our standard assay buffer and in Dulbecco's PBS, pH 7.2, supplemented with 5% (w/v) D-glucose (PBSG) (Fig. 2b). As observed previously (Fig. 1a), there were clear effects of the presence of iron in the growth medium in down-regulating WST-1 activity, and the majority of the activity was *CaFRE10*-dependent under both assay conditions. However, there was no difference in the rate of WST-1 reduction between cells assayed in standard assay buffer compared to those assayed in PBSG. Addition of 50 mM citrate to PBSG shifted the pH to 7.3 and decreased the rate of WST-1 reduction by three- to fourfold (Fig. 2b). The reason for this decrease is not known. Taken together, these results suggest that citrate does not mediate the cellular reduction of WST-1.

C. albicans* MTT intracellular reductase activities: iron regulation but no role for *CaFRE10

MTT has greater lipophilicity than XTT and WST-1, and is likely reduced by intracellular reductants (Berridge *et al.*, 2005). Examination of MTT reduction with whole cells revealed little to no difference in MTT reductase activity between wild-type (Fig. 3a, W) and the *CaFRE10*^{-/-} mutant (Fig. 3a, Δ), indicating that the reduction of MTT was not dependent on CaFre10p. Light microscopy of cells indicated that the reduced MTT product was associated with intracellular membrane structures, following the reductase assay (data not shown). Despite the lack of dependence on *CaFRE10*, MTT reduction was still iron-regulated. Three independently isolated *C. albicans* strains, SC5314 (W), WO-1 [White (Wh) and Opaque (Op) morphologies], and CBS5736 (W2), showed 5–10-fold more MTT reductase activity in iron-deprived compared with iron-replete cells. Both the MTT and the WST-1 reductase activities of the high-frequency switching strain WO-1 were less than those of strains SC5314 and CBS5736. Strain WO-1 was examined further after confirming the morphology of subtypes by microscopic examination of 200–500 cells from representative cultures. Under these mild conditions of iron limitation, there was little effect of iron on the switching process. The opaque cells had slightly, but consistently, higher MTT and WST-1 reductase activities.

MTT reductase activity in *S. cerevisiae* (Fig. 3a, Sc) was considerably less than that in *C. albicans*, and, like *C. albicans*, MTT reduction in *S. cerevisiae* was independent of the externally directed ferric reductases, encoded by *ScFRE1* and *ScFRE2* (data not shown). In contrast to *C. albicans*, there was no evidence that the low amount of MTT reduction observed in *S. cerevisiae* was iron-regulated. Deletion of mtDNA in *S. cerevisiae* (ρ^0) caused a small but statistically significant (Student's *t* test, *P*=0.0002) increase in the rate of MTT reduction, which was diminished when the cells were grown in the presence of iron (Fig. 3a). WST-1 reduction rates observed in *S. cerevisiae* were unaffected by

Fig. 3. MTT reductase activity is iron-regulated but independent of *CaFRE10*. *C. albicans* strains SC5314 [wild-type (W)], BCa15-2 [*CaFRE10*^{-/-} (Δ)], WO-1 [white phase (Wh)], WO-1 [opaque phase (Op)], CBS5736 [wild-type (W2)] and *S. cerevisiae* strains YPH499 (Sc) and YPH250 ρ^0 (ρ^0) were grown in low-iron defined medium (open bars) and the same medium supplemented with 50 μ M ferric ammonium sulfate (solid bars) at 30 °C. At exponential phase, the cells were washed and assayed for MTT (a) and WST-1 (b) reductase activities. Data are means $\pm$ SD for five independent cultures.

mtDNA status; however, the low values may preclude firm conclusions from being made (Fig. 3b). When we measured respiration directly in *C. albicans*, we did not observe any difference in the rate of oxygen consumption between the wild-type and the *CaFRE10*^{-/-} mutant strains (data not shown). These data suggest that the reductase activity encoded by *CaFRE10* is independent of mitochondrial respiration.

DISCUSSION

Tetrazolium salt reductase assays have been used to assess the effectiveness of antifungal drugs by providing rapid, real-time and quantitative assay measurements of cell-dependent activities. The question addressed in this paper is, what enzyme(s) is responsible for XTT reduction when used to assess fungal and, in particular, *C. albicans* viability/biomass? Using a genetic approach, we have demonstrated that the cell surface ferric reductase activity of *C. albicans*, encoded by *CaFRE10*, was primarily responsible for the reduction of XTT and WST-1 when *C. albicans* was grown in unbuffered medium.

The ability of *C. albicans* CaFre10p to reduce such structurally different substrates raises the possibility that an

intermediate electron carrier performs reduction of ferric iron and tetrazolium salts. The standard XTT assay utilizes menadione, and protocols using WST-1 include 1-mPMS (Meletiadiis *et al.*, 2001; Scarlett *et al.*, 2005). In our assays, omission of these two electron carrier intermediates from their respective assays led to only small decreases in tetrazolium reduction rates (20% or less). Citrate is a major component of the reductase assay buffer (Avron & Shavit, 1963; Dancis *et al.*, 1990; Lesuisse *et al.*, 1987); however, it was not required for reduction of WST-1. The presence of SOD did decrease the rate of tetrazolium reduction. However, most XTT and WST-1 reductase activity was SOD-independent. *C. albicans* is flavinogenic, and flavin secretion is increased when *C. albicans* is grown under iron-limiting conditions (Burkholder, 1943; Knight *et al.*, 2002). In our study, the cells were washed before the enzyme assays; however, it cannot be excluded that sufficient flavin was secreted by *C. albicans*, during the 10–15 min assay, to mediate electron transfer from the reductase enzyme to the tetrazolium salt. The possibility that the ferric reductases can transfer electrons directly to the tetrazolium salts remains to be tested.

The expression of *CaFRE10* is highly regulated by environmental conditions. Under iron-limiting conditions, *CaFRE10* mRNA levels are substantially elevated (Knight *et al.*, 2002). The increase in *CaFRE10* expression under iron-limiting conditions is reflected in an increase in cell surface ferric reductase activity and, as shown in this study, an increase in the rate of reduction of XTT and WST-1. However, iron is not the only environmental factor that influences cell surface reductase activity. Changing the pH to near-neutral conditions increased cell surface reductase activity when ferric iron and XTT were electron acceptors, but the activity was independent of *CaFRE10*. Nonetheless, at near-neutral pH, cell surface reductase activity was still repressed by environmental iron. There are a number of *FRE* homologues in *C. albicans*. Microarray data demonstrate that *CaFRE10* is down-regulated at alkaline pH and that the expression of four other *CaFRE* homologues [*CaFRE1* (orf19.8848), *CaFRE2* (orf19.8849), *CaFRE7* (orf19.6139), *CaFRE9* (orf19.3538)] are upregulated at alkaline pH (Bensen *et al.*, 2004). Although the gene(s) responsible for encoding the cell surface reductase at neutral and alkaline pH still needs to be identified, the regulated expression of these genes encoding ferric reductase homologues may have strong effects on tetrazolium reduction activity.

The tetrazolium salt MTT differs from XTT and WST-1 in that it is cell-permeable and can cross the plasma membrane (Berridge *et al.*, 2005). Here, we have shown that the reduction of MTT in *C. albicans* was independent of the cell surface reductase encoded by *CaFRE10*. This observation is in agreement with the intracellular site of MTT reduction demonstrated in the rat B12 neural cell line (Liu *et al.*, 1997). However, MTT reductase activity in *C. albicans* was iron-regulated. As stated above, there are a number of *FRE* homologues in *C. albicans* of unknown cellular location,

and it is possible that one or more of these is responsible for the intracellular reduction of MTT. MTT reductase of *C. albicans* was much more active than that of *S. cerevisiae*, and only the *C. albicans* MTT reductase was iron-regulated. Perhaps *C. albicans* possesses a distinct regulatory circuit that links iron to NAD(P)H levels. The high reductase activities of *C. albicans* may distinguish it from non-pathogenic *S. cerevisiae*. Finally, the use of tetrazolium salts as indicators of *C. albicans* viability/biomass must be re-evaluated in view of the fact that the enzymes that catalyse the reduction of these compounds are highly regulated by growth conditions.

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