Role of the component Fre1p of the plasma membrane ferric reductase on the azo reductase activity of intact *Saccharomyces cerevisiae* cells

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Running title: Azo reductase activity in S. cerevisiae

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ABSTRACT

Unspecific bacterial reduction of azo dyes is a widely studied process in correlation with the biological treatment of coloured waste waters but the enzyme system associated to this bacterial capability has never been positively identified. Several ascomycete yeast strains display similar decolourising activities. The yeast-mediated process requires an alternative carbon and energy source and is independent of previous exposure to the dyes. When substrate dyes are polar their reduction is extracellular, strongly suggesting the involvement of an externally-directed plasma membrane redox system. The present work demonstrates that, in *Saccharomyces cerevisiae*, the ferric reductase system participates in the extracellular reduction of azo dyes. The *Saccharomyces cerevisiae* mutant strains Δ*fre1* and Δ*fre1*Δ*fre2*, but not Δ*fre2*, showed a much reduced decolourising capability, suggesting that, under the conditions tested, Fre1p is a major component of the azo reductase activity.

The abbreviations used are:

PMRS: plasma membrane redox systems; NDM: normal decolourisation medium

INTRODUCTION

Research work on biodegradative processes of azo dyes usually exploits bacterial species, either isolated or in consortia (4,36). Bacteria, under appropriate conditions (oxygen limitation, presence of substrates utilized as carbon and energy source) frequently reduce azo dyes, producing colourless amines. Nevertheless many dyes are recalcitrant to conventional wastewater treatment processes by activated sludge (4). The overall impression on this research area is that many azo dyes can be reduced (and decolourised) by a considerable number of bacterial species but, as far as we know, the enzyme responsible for the unspecific primary reduction step has never been positively identified. What is currently postulated is that reductive decolourisation of sulfonated azo dyes by living cells must occur extracellularly due to the impermeant nature of those compounds, and that the primary reductant is a cytoplasmic electron donor, presumably NAD(P)H (36).

Our own studies (30,31) have demonstrated that some non-conventional ascomycete yeasts are efficient azo dye decolourisers acting, as many bacteria, by reducing the azo bond. Dye decolourisation by yeasts is comparatively unspecific, but is affected by the medium composition, by the used yeast strain, and by parameters as pH and dissolved oxygen. It also depends on actively growing cells, being faster during the exponential growth phase, and displays an enzyme-like temperature profile, strongly suggesting its biotic nature. However, further information is required for a successful application of yeasts in a wastewater treatment process. The present work was developed to demonstrate the participation of an externally directed plasma membrane redox system (PMRS) in azo dye reduction, linking an intracellular reductant to an extracellular electron acceptor. As a required first step, it was necessary to find a model yeast strain, capable of decolourising polar azo dyes. Among the screened strains, *Saccharomyces cerevisiae* CEN.PK113-7D proved to fulfil those conditions.

In *S. cerevisiae* the most extensively explored PMRS is the ferric/cupric reductase system which participates in the high-affinity uptake of iron. This activity can be assayed through the reduction of impermeant substrates like ferricyanide, iron(III)-citrate, iron(III)-EDTA, and a variety of other ferric chelates. In this complex system the best studied components are the metalloreductases encoded by the genes *FRE1* (7) and *FRE2* (15), the *FET3/FTR1* encoding the oxidase-permease complex (reviewed in 9), the iron-dependent transcriptional regulator Aft1p (39,40) and Aft2p (3,40) and the

copper-dependent transcriptional regulator Mac1p (16,40). A potential Fe^{3+}/Cu^{2+} reductase subunit is the cytoplasmic cofactor Utr1p (1).

FRE1 and *FRE2* encode plasma membrane proteins (7,15) and are both transcriptionally activated by Aft1p, whose intracellular location is dependent on iron(III) level (42). *FRE1* activation is also controlled by Aft2p (33) and Mac1p (40). Transcription of *FRE2* depends only on iron levels (14) through Aft1p (33). The protein encoded by *FRE1* contains several transmembrane domains (7), and shares 62% sequence similarity with the $gp91^{phox}$ subunit of cytochrome b_{558} (32). The protein motifs in $gp91^{phox}$ responsible for binding FAD and NADPH are conserved in Fre1p (12,23,35). Fre1p and Fre2p together account for virtually all of the Fe^{3+}/Cu^{2+} reductase activity of yeast cells but in varying proportions, depending both on iron and(or) copper availability and on the growth phase of the cells (14,15,16). Typically *FRE2* is induced at a later stage. Fre1p and(or) Fre2p reduce external Fe^{3+} (or Cu^{2+}) prior to their uptake, mediated by Fet3p/Ftr1p, where Fet3p is a multicopper oxidase and Ftr1p the permease component (10). The cytoplasmic cofactor Utr1p in *S. cerevisiae* has recently been shown to be a NAD kinase (21) which is regarded as the only enzyme catalysing the synthesis of NADP.

The genome sequence of *S. cerevisiae* revealed the presence of five additional metalloregulated genes, *FRE3-FRE6* and *FRE7*, with sequence similarities to *FRE1* and *FRE2*. The first four are transcriptionally regulated by the iron-responsive Aft1p element and the fifth by the copper-dependentMac1p (27). Fre3p and Fre4p are potential siderophore-iron reductases (43), but the function of the remaining genes is unknown. Given their regulation pattern they may participate in iron homeostasis (*FRE5*, *FRE6*) and copper homeostasis (*FRE7*), possibly as internal metalloreductases (27).

The present work shows that the azo reductase and ferric reductase activities of yeast cells assayed in different growth phases are closely parallel, being at the highest level during the exponential growth phase. This property of ferric reductase has been described in earlier studies (6,15). Also, deletion of *FRE1* gene eliminates a major fraction of the azo reductase activity in intact cells of *S. cerevisiae* harvested in the late exponential growth phase, whereas the deletion of the *FRE2* gene has a minor effect on that activity. We believe that our results will be relevant for biotechnological applications of this activity and also for a broader understanding of the unspecific redox activities associated to the yeast plasma membrane.

MATERIALS AND METHODS

Chemicals. The azo dye used in the experiments was *m*-[(4-dimethylamino)phenylazo] benzenesulfonic acid, sodium salt, and was synthesized as described for methyl orange (13).

Yeast strains and plasmids. The yeast strains and the plasmids used in this work are listed respectively in tables 1 and 2. The cultures were maintained on slants of YPD - yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v). Growth on solid media was carried out at 30ºC.

Cell growth in liquid medium. The attenuance of appropriately diluted cell suspensions (as described in 30) was measured at 640 nm in a Spectronic 21 Bausch & Lomb using a 1 cm path length cell.

Decolourisation in liquid media. Decolourisation experiments by growing cultures of *S. cerevisiae* CEN.PK113-7D (also reported as wt strain along this work) were typically performed in 250 mL cotton-plugged Erlenmeyer flasks with 100 mL of sterile medium (normal decolourisation medium, here referred to as NDM) containing yeast extract (0.25%, w/v), glucose (2%, w/v) and 0.2 $mmol.L^{-1}$ of the tested dye in a mineral salts base of the composition previously described (37) incubated at 26ºC and 120 rpm. Whenever required, iron (III) was added to medium as the EDTA chelate, from a 100 mM stock solution in $FeCl_3$ and EDTA. For the mutant strains, which show impaired growth in our standard medium, cells were grown for 137 h on NDM supplemented with 2 mM iron (III) as the EDTA chelate. For control wild-type cells were grown in similar conditions. The cells were then harvested by centrifugation at 16.1xg, washed several times with sterile distilled water, and resuspended on NDM to produce cell suspensions with 3.8±0.2 attenuance units (4.2±0.2 $g.L^{-1}$ cell dry weight). Throughout this work, decolourising activity refers to the decolourisation capability of growing yeast cultures.

Cell counting. Cell suspensions (diluted to an attenuance of c.a. 0.5 units) were diluted 1:25000 and 1:250000. From each dilution 100 µL was spread in YPD agar plates. The plates were incubated at 37ºC for 2 days and after that time the number of isolated colonies was counted. All plates with more than 300 colonies or less than 30 were not considered. All the dilutions were prepared in triplicate.

Ferric reductase assay. Cells were grown for *c.a*. 6 hours in NDM, harvested by centrifugation, washed twice with sterile distilled water and resuspended in assay buffer, consisting of 0.05 M sodium citrate pH 6.5 with 5% glucose, at a density of *c.a.* 1.3±0.1

attenuance units (1.4±0.1 $g.L^{-1}$ cell dry weight). The assays were performed in triplicate at two different cell densities obtained with either 780 μL of suspension or 390 μL of suspension plus 390 μL of assay buffer. The cell suspensions were pre-incubated for 10 min at room temperature. The final assay mixtures contained, in a total volume of 1 mL, 2 mM ferrozine ([3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine]) and 0.2 mM iron(III) as ferric chloride. The mixtures were allowed to react at room temperature (20±2ºC) for 5 or 10 min. Cells were then harvested by centrifugation and the optical density at 562 nm was measured against a blank prepared similarly but without cells. The ferrous iron concentration was estimated by using a molar absorbance of 27900 $M^{-1}cm^{-1}$ for the iron(II)-ferrozine complex (17).

Azo reductase assays. These assays were performed as the ferric reductase assays but using acetate buffer 0.05 M pH 4.0 and 5% glucose. The assay mixture contained a cell suspension of 1 or 2 attenuance units (1.1±0.1 or 2.2±0.1 $g.L^{-1}$ cell dry weight) and 0.05 mM dye, and was allowed to react for 15 to 20 min. Within this period the decrease in absorbance was linear with time. The optical density of the final supernatants was read at dye $\lambda_{máx}$ (461 nm). The amount of dye reduced was determined from a molar absorbance of 21440 $M^{-1}cm^{-1}$, obtained from a calibration curve. Throughout this work, azo reductase activity refers to the results of activity assays within a short period of time, being expressed as $\mu mol.(g\ cell\ dry\ weight.min)^{-1}$.

Transformation of S. cerevisiae cells. Transformation of *S. cerevisiae* cells was done by the LiAc/SS-DNA-PEG method (18). When required transformants were recovered at 30ºC in YPD medium for 4 hours before plating on YPD solid medium containing either 200 $mg.L^{-1}$ geneticin (G418 from Life Technologies) or 30 $\mu g.L^{-1}$ phleomycin (CAYLA, Toulose, France). Transformants were obtained after 2-3 days of incubation at 30ºc. To purify transformants from background each large colony was re-streaked on fresh YPD-geneticin or YPD-phleomycin plates. Only those clones that grew after the double selection were further analysed as potentially correct transformants, by analytical PCR as described by Kruckeberg (22).

Cloning of the FRE1 and FRE2 genes. *FRE1* gene was amplified by PCR with the Pfu Turbo DNA polymerase (Stratagene), using the primers Fre1forw and Fre1rev and genomic DNA isolated from *S. cerevisiae* CEN.PK. The PCR fragment was cloned into the plasmid pGEM®-T Easy vector (PROMEGA), originating the plasmid pSP1 (table 2). The primers Fre2forw and Fre2rev were used to amplify *FRE2* gene, following the

same procedure as described for *FRE1* gene. The PCR product was cloned in pGEM®-T Easy vector originating the plasmid pSP2 (table 2). DNA cloning and manipulation were performed according to standard protocols (34).

FRE1 knock-out. The *S. cerevisiae* Y04163 strain deleted in the gene *FRE1* (*YLR214W*) was obtained from the Euroscarf collection. Two primers, A-YLR214W and D- YLR214W (table 3) were used to amplify by PCR the *YLR214W::KanMX4* allele of the *S. cerevisiae* strain Y04163. The PCR product was used to transform wt cells. Cells were plated on YPD solid medium containing 200 $mg.L^{-1}$ geneticin. Successful integration of the *YLR214W::KanMX4* cassette was scored by presence of the *YLR214W::KanMX4* band (2352bp) and absence of the *YLR214W* wild-type band (2796bp) following analytical PCR on whole cells using the same primers. Internal primers to the kanamicine cassette (K2 and K3, see table 3) were also used to reconfirm the disruption. This strain was named SP1.

FRE2 knock-out. The procedure followed to disrupt the gene *FRE2* (*YKL220C*) *was* similar to the one described above. Primers, A-YKL220C and D-YKL220C (table 3) were used to amplify by PCR the *YKL220C::KanMX4* allele in the *S. cerevisiae* strain Y07039. The PCR product was used to transform the *S. cerevisiae* CEN.PK strain and correct integration of the cassette was scored by presence of the *YKL220C::KanMX4* band (2323bp) and absence of the YKL220C wild-type band (2842bp) following analytical PCR on whole cells using the same primers. This strain was named SP2.

FRE1/FRE2 double knock-out. The vector pAG32, containing the hygromycin resistance gene *HphMX4*, was digested with the restriction enzymes *Bgl*II and *Eco*RV. The digested DNA was used to switch the selective marker of the gene replacement casette in *S. cerevisiae* Y07039 from *KanMX4* to *HphMX4*, resulting in strain SP3. The replacement of the *KanMX* for the *HphMX4* was confirmed with PCR. SP3 chromosomal DNA was used to amplify the *YKL220C::HphMX4* casette, which was used to transform the SP1 (already carrying the *YLR214W::KanMX4*) resulting in the double mutant, SP4.

RNA analysis. Total cellular mRNA was prepared from yeast cells grown for 6 hours in NDM, electrophoresed on 1.5% (w/v) agarose MOPS-formaldehyde gels (29) and blotted to nylon membranes by vacuum transfer. Hybridisation was carried out using a fragment of 718 bp *Pst* I from pSP1 as a probe for *FRE1* or a fragment of 682 bp *Hind*III from pSP2 as a probe for *FRE2*. The probes were labelled according to standard

procedures (34). Densiometer scanning was performed using the Integrated Density Analysis program from the EagleSight® Software, version 3.2 (Stratagene, CA).

Construction of the pSH65-FRE1 vector. The ORF of *FRE1* was amplified by PCR with the primers CMPfre1forw and CMPfre1rev. CMPfre1forw contains one BamHI site and the CMPfre1rev contains one SalI site which were used for cloning the *FRE1* ORF in the vector pSH65 (20) using the same restriction sites. The *FRE1* ORF was directionally cloned between the GAL1,10 promoter and the CYC1 terminator in the vector pSH65, which is a CEN6/ARSH4 low-copy number vector carrying the ble^r phleomycin resistance gene for selection in yeast. Correct clones were verified by sequencing. A clone named pSP3 (table 2) was selected for further studies.

Transformation of the Δfre1 with the plasmid pSP3 (pSH65-FRE1). Cells of the strain SP1 were transformed with the plasmid pSP3 and placed on YPD solid medium containing 30 $\mu g.L^{-1}$ phleomycin. Ten colonies were checked by analytical PCR using the primers GAL1p_c and CMPfre1rev. The method described by the "The SixPack Guidelines" of the EUROFAN project was used. The GAL1p_c and the CMPfre1rev forms a 2.1 kb PCR product only if the *FRE1* ORF is present in the correct orientation with respect to the GAL1,10 promoter in pSH65. One of the positive strains was named SPcmp-*FRE1* (table 1) and was used in further studies.

RESULTS

Decolourization by growing yeast cultures. Growing cultures of *S. cerevisiae* completely decolourised the tested azo dye in c.a. 8.5 h. Figure 1(a) illustrates the yeast cells growth curve, and the pH variation and dye absorbance in the supernatant medium. A diauxic growth was observed, with a specific growth rate of 0.175 h^{-1}, when growing in glucose, and of 0.013 h^{-1} after switching to ethanol utilization. The decolourisation progress was unaffected by previous exposure of the cells to the dye (results not shown). Similar observations have been described earlier for *Candida zeylanoides* (31) and *Issatchenkia occidentalis* (30). The confirmation that colour loss was due to the reductive cleavage of the azo bond in the dye molecules was provided by the detection of the related aromatic amines by HPLC analysis, as shown in a previous work (31).

The effect of the growth phase on specific ferric and azo reductase activities was determined by assaying cells harvested from growing cultures at different incubation times. The results are shown in figure 1B, and despite the difference in the absolute values, the two curves are closely parallel at all times. Both have an activity peak in the late exponential growth phase, which is also when the fastest decrease of dye concentration in the incubation medium is observed.

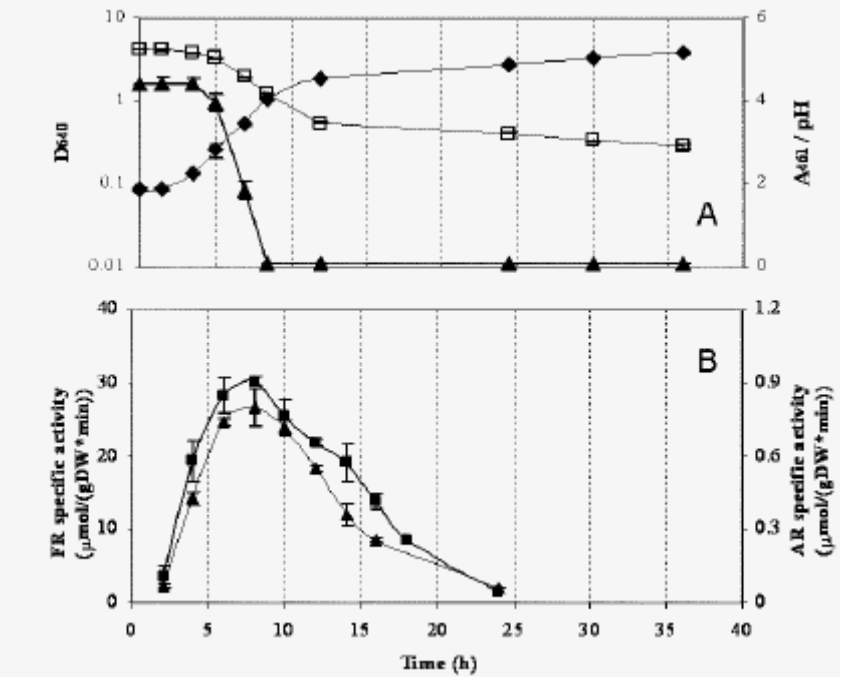

Figure 1. **Decolourisation progress and effect of growth stage on ferric reductase and azo reductase specific activities.** (A) Time course of cell growth, measured as attenuance at 640nm (D_{640}; ♦), pH variation (pH; □) and progress of decolourisation, measured as dye absorbance at 461 nm (A_{461};▲). *S. cerevisiae* was grown at 26ºC and

120 rpm, in normal decolourisation medium containing 0.2mM dye. (B) Variation of ferric reductase (FR; ■) and azo reductase (AR; ▲) specific activities in cells of *S. cerevisiae* harvested at the specified times, expressed as μmol.(g cell dry weight)$^{-1}$.min^{-1}. The cells were grown in normal decolourisation medium at 26ºC and 120 rpm.

Effect of iron concentration on specific ferric and azo reductase activities. The progress of decolourisation by growing cultures was measured in incubation media with different iron (III) concentrations, supplied as the EDTA chelate. Increasing iron concentrations resulted in a much delayed decolourisation. As seen in figure 2A, total decolourisation required over 50 h in the presence of 1.0 mM iron (III), in contrast with the 8.5 h required in NDM without iron addition. In media containing 2.5mM iron(III) dye concentration decreased only *c.a.* 20% in 75h. For concentrations above 2.5mM iron(III) we observed precipitation of the iron in the medium. The reduced decolourising activity of the cells grown at higher iron concentrations was not due to impaired growth or loss of cell viability since cell counting in aliquots of the different cultures, collected after 28 h of growth, produced identical numbers of viable cells.

Azo and ferric reductase activities were also measured in cells harvested from growth media with different iron concentrations, after 6 hours of growth. Cells were collected at this point because of the peak activity of both enzymes around this time. The results in Figure 2B show that the production of both activities was repressed by iron, in a concentration-dependent manner: azo reductase activities are reduced to *c.a.* 20% at 1 mM iron and to 2% at 2.5 mM iron, despite the growth stimulation at higher Fe concentrations (data not shown). These observations point to an additional link between the two activities.

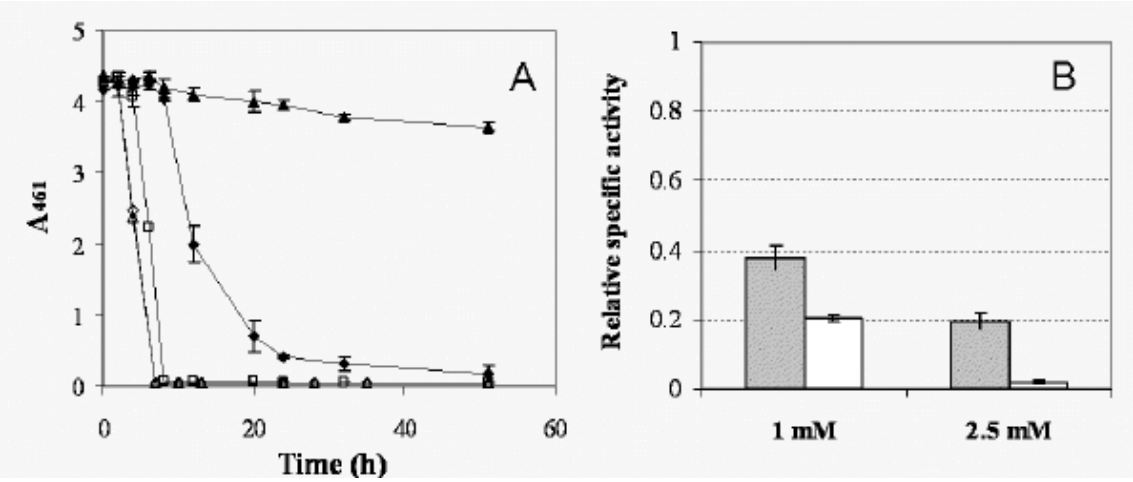

Figure 2. **Iron(III)-dependent decolourisation and activities of ferric reductase and azo reductase**. (A) Time course of dye decolourisation in the presence of 1.0mM (♦) and 2.5mM (▲) iron (III). Cells were grown at 26ºC and 120 rpm in normal decolourisation medium with 0.2mM dye and iron was supplied as the EDTA chelate to the specified concentrations. Control experiments were performed without iron addition to the medium (□) and in media supplemented with EDTA, either at 1mM (◊) or 2.5mM (△). The effect was followed by measuring dye absorbance at 461 nm (A_{461}). (B) Specific activity assays of ferric reductase (*grey bars*) and azo reductase (*white bars*) were performed with cells harvested after 6 hours growth on normal decolourisation medium at 26ºC and 120 rpm. Growth media contained either 1.0mM or 2.5mM iron(III). Specific activities were calculated relative to cells grown without additional iron(III). Activities were calculated relative to cells grown without additional iron(III). Error bars are the standard deviation from three independent determinations.

Effect of deletions of FRE1 and FRE2 genes on the activities of ferric and azo reductases. The mutant strains of *S. cerevisiae* Δ*fre1, Δfre2 and* Δ*fre1*Δ*fre2* have impaired growth in iron-deficient media. In order to overcome this problem, decolourisation assays with the mutant strains were performed at high density suspensions of pre-grown cells, as described in Materials and Methods. Under these conditions both the wt strain and the Δ*fre2* mutant achieved complete decolourisation in *c.a.* 5 hours. Therefore deletion of the *FRE2* gene has a negligible effect in the decolourisation process in our experimental conditions. In contrast, the Δ*fre1* and Δ*fre1*Δ*fre2* strains showed a much reduced decolourising activity, requiring more than 45 hours to completely remove the colour from the medium (figure 3A). The azo reductase activity assays with the different strains allowed similar conclusions. As seen in figure 3B, the specific activity in the Δ*fre2* mutant reached the same order of magnitude (as compared to the wild type), whereas those in Δ*fre1* and Δ*fre1*Δ*fre2* strains was negligible. The ferric reductase assays produced very similar results, as seen in figure 3B. These results demonstrate the importance of the *FRE1* gene product in the decolourising activity of the yeast cells.

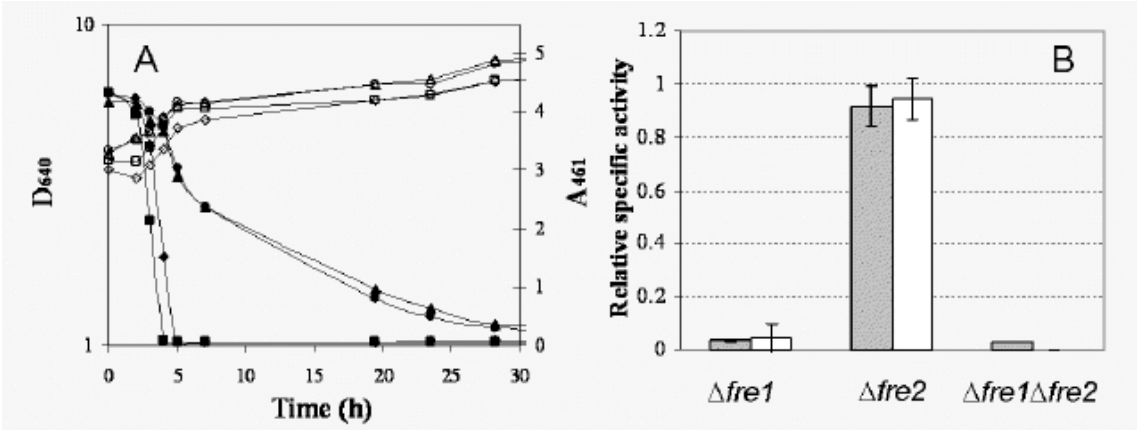

Figure 3. **Deletion of *FRE1* and *FRE2* genes affects decolourisation progress and ferric reductase and azo reductase activities.** (A) Cells were grown at 26ºC and 120 rpm on normal decolourisation medium with 0.2mM dye. Cell growth was measured as attenuance at 640nm (D_{640}; *open symbols*) and decolourisation progress was assessed by dye absorbance at 461 nm (A_{461}; *closed symbols*): wild type (◊, ♦), Δ*fre1* (△, ▲), Δ*fre2* (□, ■) and Δ*fre1*Δ*fre2* (○, ●). (B) Activities of the ferric reductase (FR; *grey bars*) and azo reductase (AR; *white bars*) of *FRE* mutant strains were calculated relative to cells of the reference strain, all grown on NDM at 26ºC and 120 rpm and harvested after 6 hours growth.

***FRE1* expression in *S. cerevisiae*.** The expression of *FRE1* was followed by Northern-blot analysis (figure 4). In cells of wild-type strain *S. cerevisiae* CEN.PK, grown in the absence of added iron, a strong mRNA signal against a *FRE1* probe was revealed, proving the expression of this gene. Wild-type cells, grown in the presence of added iron showed decreased *FRE1* mRNA levels with increasing iron concentration in the range between 1.0 and 2.5 mM. Therefore, iron seems to regulate the expression of *FRE1* gene. As expected, in cells of *S. cerevisiae* Δ*fre1* and Δ*fre1*Δ*fre2* deletion strains, no *FRE1* mRNA was detected.

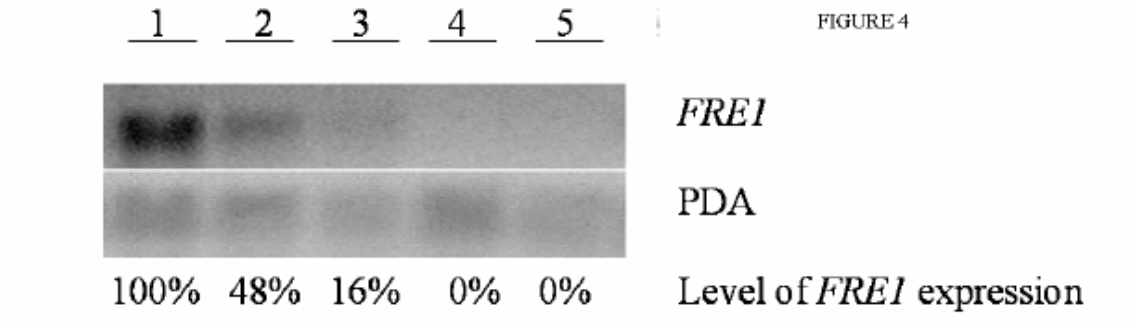

Figure 4. **Northern blot analysis of *FRE1* transcriptional level.** Cells used for RNA extraction were harvested after 6h growth on normal decolourisation medium at 26ºC and 120 rpm, with or without iron addition. Each lane contained 20 μg of total RNA and PDA1 (38) served as internal standard. Lane 1-wt; lane 2- wt with 1mM iron(III) added to the growth medium; lane 3- wt with 2.5mM iron(III) added to the growth medium; lane 4- Δ*fre1*; lane 5- Δ*fre1*Δ*fre2* . The percentage of *FRE1* expression (average of two independent experiments) is relative to wt strain, grown in normal decolourisation medium without externally added iron.

Recovery of the *FRE1* activity. To confirm that in our experimental conditions the recovery of the azo reductase activity is mainly associated with *FRE1*, the progress of decolourisation was followed in cultures of the strains wt, Δ*fre1* and Δ*fre1* transformed with the plasmid pSP3 containing *FRE1* under the promoter GAL1,10. The cells were grown in media with 20g.L^{-1} galactose as carbon source, for activation of the GAL1,10 promoter. As seen in figure 5, *FRE1* gene complemented the phenotype of *S. cerevisiae* Δ*fre1* cells recovering the ability to grow in medium without externally added iron, following a pattern similar to the one observed in the wt strain. In this assay the wt and Δ*fre1* strains behaved as expected regarding the ability of decolourisation, with a total removal in the wt and a negligible removal in the mutant strain. The transformed strain Δ*fre1*(pSP3), although with a small delay in the starting of the decolourisation, was able to fully decolourise the dye. This small difference could be due to distinct regulatory properties of the two promoters. These experiments provide the evidence that *FRE1* is responsible for the azo reductase activity of the intact yeast cells in our operational conditions.

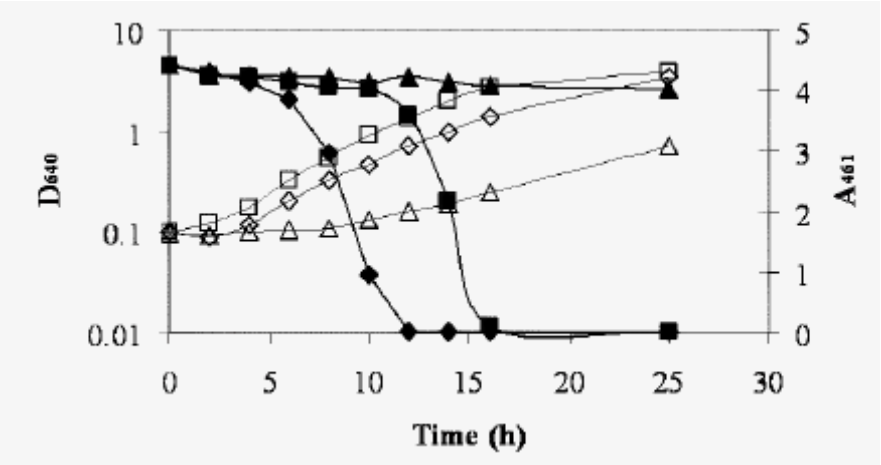

Figure 5. **Reconstitution of the *FRE1* activity.** Cells were grown at 26ºC and 120 rpm on normal decolourisation medium with 0.2mM dye and 20g.L^{-1} galactose as carbon source for activation of the GAL1,10 promoter. Cell growth was measured as attenuance at 640nm (D_{640}; *open symbols*) and decolourisation progress was assessed by dye absorbance at 461 nm (A_{461}; *closed symbols*): wild type (◊, ♦), Δ*fre1* (△, ▲) and SPcmp-*FRE1* (□, ■).

DISCUSSION

Plasma membrane redox systems are ubiquitous, being expressed in all living cells including bacteria and cyanobacteria, yeasts, algae and also in plant and animal cells (8,26). These systems are linked to several vital cellular functions, including growth control, iron uptake, apoptosis, bioenergetics, transformation and hormone responses (2,5,28). Some of these roles may be linked to the maintenance of appropriate $NAD(P)^+$/NAD(P)H cytoplasmic ratios. In fact, an increase in the glycolytic flux, leading to an accumulation of NADH in the cytoplasm, induces an increase of PMRS activity (28). A number of such systems has been described, such as NADH:ascorbate free radical oxidoreductase, NADH:ubiquinone oxidoreductase and ferric reductase, among others (26,28). However it is not clear whether different phenomenological enzyme activities correspond to different PMRS. On the contrary, it is generally accepted that several PMRS are multifunctional (5,8,28).

The *FRE1*-dependent ferric reductase activity of intact yeast cells is inversely regulated by iron (III) concentration, through the transcriptional activators Aft1p and Aft2p (33,42). Our decolourisation experiments in media containing additional iron revealed a considerable increase in the time required for complete dye removal, and a negative effect of iron (III) in the azo reductase activity of yeast cells. Ferric reductase activities also decrease, as expected, but the effect of increased iron concentrations is more pronounced in the azo reductase activities.

Both ferric reductase (23) and the yeast azo reductase display an activity peak in the exponential growth phase. This is not an unexpected observation, since many enzymes involved in cell growth have peak activities in this phase, when concentrations of intracellular reductants are also high.

The use of the strains defective in the genes encoding for structural components of the transmembrane ferric reductase, *FRE1* and *FRE2*, unequivocally demonstrated that Fre1p is a major component of the azo reductase system. In contrast Fre2p had a reduced importance in azo reduction, at least under our assay conditions. Our observation is in agreement with works reporting that the *FRE1* gene accounts for 80 to 98% of the ferric reductase activity (6,7). Nevertheless, growing cultures of the Δ*fre1* strain and of the double deleted mutant still showed a low decolourising capability. A residual ferric reductase activity has been explained by postulating the existence of an excreted reductase activity (15) which, however, has never been described. An alternative explanation has been provided by Lesuisse and colleagues (25), who have

shown that the excretion of anthranilic and 3-hydroxyanthranilic acids was correlated with the extracellular ferric reductase activity. Whether those or other extracellular reductants participate in azo dye reduction requires further investigation. The insignificant participation of Fre2p in the ferric and azo reductase activities measured in this work (cells harvested after 6 hours growth) is probably due to the fact that the *FRE2* gene is expressed primarily after 8-10 hours of growth, whereas the expression of *FRE1* is highest in cells grown for up to 6 hours (14). Therefore the effect of *FRE2* was not investigated at the present stage of our work.

It must be taken into account that the ferric reductase activity of intact yeast cells does not depend exclusively on one or more transmembrane proteins encoded by *FRE* genes. The *in vivo* association of the Fre1p component to the NAD phosphorylating kinase Utr1p (21) is now generally accepted, since increased ferric reductase activity is observed only when both *FRE1* and *UTR1* are overexpressed together (23). It has therefore been suggested that Utr1p is the supplier of NADP to the ferric reductase system (26). This is also consistent with the existence of NADPH binding motif in Fre1p (12,23,35), suggesting that NADPH is the electron donor for iron reduction.

In conclusion, this work strongly suggests that the Fre1p-dependent reductase system of the yeast plasma membrane is an important component of the azo reductase activity in intact cells of *S. cerevisiae* harvested between mid and late exponential growth phase. Further information on the azo reductase system will be provided by examining the effect of known inhibitors of the ferric reductase, by establishing the nature of the electron donor and by searching other components affecting the *in vivo* fully functional system. For example, it has been demonstrated that the ferric reductase activity in isolated plasma membranes is due to a NADPH dehydrogenase (diaphorase) activity and that Fre1p, *per se*, has no reductase activity (23). Additionally it has been that activation of the *in vivo* ferric reductase system requires the integrity of the RAS/cAMP pathway (24). Interestingly, among several laboratory strains of *S. cerevisiae* the only strain with decolourising activity was the CEN.PK 113-7D, which has a mutation on the *CYR1* gene encoding the enzyme adenylate cyclase (37).

Acknowledgments – P. Ramalho gratefully acknowledges a scholarship from the European BIOEFTEX Project. The authors thank the technical help of Sónia Barbosa in the Northern-blot experiments and the expert help of Dr. Björn Johansson in all stages

of this work. P. R. would like to thank Professor André Gouffeau for a fruitful discussion and critical reading of the manuscript.

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541 **TABLES**

542 TABLE 1

543 *Saccharomyces cerevisiae strains used in this work*

Strain	Genotype	Reference
CEN.PK 113-7D	Wt (MATa, MAL2-8c SUC2)	11
Y04163	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; *YLR214W::KanMX4*	Euroscarf
Y07039	BY4741; Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; *YKL220C::KanMX4*	Euroscarf
SP1	Δ*fre1* (CEN.PK *YLR214W::KanMX4)*	This work
SP2	Δ*fre2* (CEN.PK *YKL220C::KanMX4)*	This work
SP3	BY4741; *YKL220C::HphMX4*	This work
SP4	Δ*fre1*Δ*fre2* (CEN.PK *YLR214W::KanMX4 YKL220C::HphMX4)*	This work
SPcmp-*FRE*1	Δ*fre1*(pSP3) (CEN.PK *YLR214W::KanMX4* + plasmid pSP3)	This work

544

545

TABLE 2

546

Plasmids used in this work

Plasmids	Reference
pSP1 (*FRE1* in pGEM)	This study
pSP2 (*FRE2* in pGEM)	This study
pAG32	19
pSH65	20
pSP3 (*FRE*1 in pSH65)	This study

547

548

TABLE 3

549

Oligonucleotides used for cloning, gene deletion and verification by PCR

Name	Sequence
A-YLR214W	AAAAATGTATTTAGGTTGCTTGACG
D-YLR214W	TATGAATTAAGGTTAGTGACGAGGC
A-YKL220C	ACAGGAAAACAAGTAAATTTTGACG
D-YKL220C	CAATTAACGTTTCATAAAATTTGCC
Fre1forw	ATGGTTAGAACCCGTGTATTATTC
Fre1rev	TTACCATGTAAAACTTTCTTC
Fre2forw	ATGCATTGGACGTCCATCTTG
Fre2rev	TCACCAGCATTGATACTCTTC
K2	CGATAGATTGTCGCACCTG
K3	CCATCCTATGGAACTGCCTC
CMPfre1fw	CATGGATCCAAAATGGTTAGAACCCGTG
CMPfre1rev	CATGTCGACTTACCATGTAAAACTTTCTTC
GAL1p_c	ATTGTTAATATACCTCTATACTTTAAC

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