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Research Article

Isolation of a *Candida albicans* gene, tightly linked to *URA3*, coding for a putative transcription factor that suppresses a *Saccharomyces cerevisiae aft1* mutation

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Abstract

A pathogen such as *C. albicans* needs an efficient mechanism of iron uptake in an iron-restricted environment such as is the human body. A ferric-reductase activity regulated by iron and copper, and analogous to that in *S. cerevisiae*, has been described in *C. albicans*. We have developed an in-plate protocol for the isolation of clones that complement an *aft1* mutation in *S. cerevisiae* that makes cells dependent on iron for growth. After transformation of *S. cerevisiae aft1* with a *C. albicans* library, we have selected clones that grow in conditions of iron deficiency and share an identical plasmid, pIRO1, with a 4500 bp insert containing the *URA3* gene and an ORF (*IRO1*) responsible for the suppression of the iron dependency. *IRO1* does not show homology with *AFT1* or with other sequences in the databases. Northern analysis demonstrates constitutive expression of *IRO1*. CAI4, a *C. albicans* strain isolated as Δ*ura3*, also has a deletion of the 3′ half of *IRO1*, and displays in YNB medium similar phenotypic characteristics to *S. cerevisiae aft1* mutant strains. Therefore, we consider *IRO1* as a gene of *C. albicans* involved in the utilization of iron. However, in extreme conditions of iron deprivation, CAI4 seems to activate alternative mechanisms of iron uptake that allow a better growth than the wild strain SC5314. Analysis of its predicted protein sequence is in agreement with a role of Iro1p as a transcription factor. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: ***Candida albicans*; iron uptake; transcription factor**

Introduction

Candida albicans is a polymorphic fungus, its main morphological forms being yeast and mycelia. In recent years, the occurrence of systemic infections due to *C. albicans* has been observed more frequently, as a consequence of increasing immunodeficiencies of different origins, such as AIDS, antileukaemic treatment or therapeutic immuno suppression in transplant patients and others (Beck-Sague and Jarvis, 1993; Edwards, 1990; Neil and Ampel, 1996; Sobel and Vazquez, 1990).

C. albicans belongs to the imperfect fungi, lacking a known sexual cycle and having a diploid or aneuploid genetic constitution, and so mutants are difficult or almost impossible to obtain when phenotypes are not selected in a simple way. Consequently, *C. albicans* is not suitable for genetic studies. Under these circumstances, molecular approximation can be advantageous (Fonzi and Irwin, 1993) and within this framework we have begun to study the molecular components involved in iron uptake by *C. albicans*.

Most living beings need iron as a co-factor essential for the activity of multiple catalytic proteins. This element is not easily available in the host, which sequesters this element as a defence mechanism. Because of this, pathogenic microorganisms are dependent on extremely effective systems of iron uptake (Kontoghiorghes and

Weinberg, 1995; Weinberg, 1998) and these systems represent important virulence factors. For this reason, the mechanisms of iron uptake in different bacterial pathogens are currently being studied (Guerinot, 1994). The bacterial mechanisms differ from eukaryotic systems by the presence of siderophores, as in *Escherichia coli* (Bagg and Neilands, 1987), or by the ability to acquire iron by capturing mammalian proteins, as in *Neisseria* (Genco and Desai, 1996).

Although a clear relationship between iron uptake and virulence has not been established, different observations attest to the importance of iron uptake in *C. albicans* virulence (Fratti *et al*., 1998). It is known that salivary lactoferrin is one of the factors that reduce the infective capacity of this fungus in the oral cavity (Palma *et al*., 1992; Xu *et al*., 1999). Human serum inhibits the growth of *C. albicans*, fundamentally due to the presence of transferrins; proteins such as lactoferrin and ovotransferrin have similar effects to serum. On the other hand, haemoglobin addition suppresses the effect of serum and these proteins (Radke *et al*., 1994; Watanabe *et al*., 1997). These data suggest that *C. albicans* must compete for iron with these iron-complexing proteins and, therefore, iron-sequestering mechanisms must be considered as virulence factors. Furthermore, the adherence to epithelial cells and formation of germ tube are also regulated by iron availability. A change in the electrophoretic pattern of cell wall proteins, as well as in antigenic determinants, has been reported in response to iron concentration (Abe *et al*., 1985; Paul *et al*., 1989; Sweet and Douglas, 1991).

The importance of iron for the development of life, together with the potential toxicity of an excess of this element, gave rise to the development of complex mechanisms that regulate the uptake of iron from media. In *S. cerevisiae*, highly specific mechanisms of iron uptake and transport have been described (for reviews, see Eide, 1998; Guerinot, 1994; Stearman *et al*., 1996). These mechanisms are regulated at the transcriptional level by the product of *AFT1* in response to iron (Yamaguchi-Iwai *et al*., 1995, 1996; Casas *et al*., 1997). In *C. albicans* the mechanisms of iron uptake are not well characterized, although this fungus probably uses a system of iron uptake through siderophores (Holzberg and Artis, 1983; Ismail and Lupan, 1986; Ismail and Bedell, 1986; Minnick *et al*., 1991). A mechanism of iron acquisition from blood cells which exploits the host complement system, based in a *C. albicans* complement receptor-like molecule, has been reported (Moors *et al*., 1992). *C. albicans* possesses ferric-reductase activity, regulated by iron and copper (Morrissey *et al*., 1996), that would be analogous to the *FRE1*- and *FRE2*-associated activities in *S. cerevisiae*. The latter have been shown to play an important role in iron transport in this species (Dancis *et al*., 1990; Georgatsou and Alexandraki, 1994; Stearman *et al*., 1996). In this work, we employ an *S. cerevisiae* Δ*aft1* mutant to isolate and characterize *C. albicans* genes involved in iron transport.

Materials and methods

Strains, libraries and probes

S. cerevisiae strains: CML126 (*MAT***a** *leu2-3,112 ura3-52 trp1-1 his4 can1*^{R} *aft1-*Δ*5::URA3*) and CML128 (*MAT***a** *leu2-3,112 ura3-52 trp1-1 his4 can1*^{R}) were described in Casas *et al*., (1997). Δ*mac1*, and Δ*fre1*/Δ*fre2* strains were kindly provided by Dr Despina Alexandraki (Foundation for Research and Technology – Hellas, Institute of Molecular Biology and Biotechnology). *C. albicans*: ATCC2655 (ATCC) and SC5314 (Gillum *et al*., 1984) were wild-type clinical isolates. CAI4 (Δ*ura3::*λ*imm434*/Δ*ura3::*λ*imm434*) was obtained from SC5314 by Fonzi and Irwin (1993). *C. albicans* genomic library (in YRp7 vector; Struhl *et al*.,1976) has been described previously (Nieto *et al*., 1993). *C. albicans URA3* probe has also been described previously (del Castillo Agudo *et al*., 1993). *IRO1* probe was obtained by labelling a *Hin*cII–*Hin*cII pIRO1 fragment or by PCR amplification with specific primers; the *URA3* and *IRO1* probes overlap and contain the *Eco*RV site of the *IRO1* open reading frame (ORF) (Figure 1).

Enzymes and chemicals

DNA restriction endonucleases and T4 DNA ligase were supplied by Boehringer-Mannheim, Pharmacia or Promega. *Taq* DNA polymerase was purchased from Pharmacia. Ferrozine, α*α*′-dipyridil (DPD), ferrichrome and fluorochromes rhodamine 123 (RH123) and dihydroethidine (HE), were purchased from Sigma (St. Louis, MO, USA).

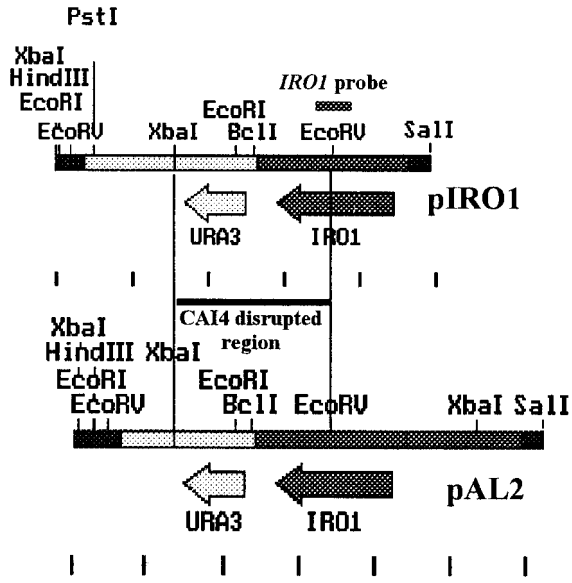

Figure 1. Comparative maps of *C. albicans* DNA inserts and flanking regions (dark areas in plasmids pIRO1 and pAL2). Spaces between vertical markers = 1 kb

Media and growth conditions

C. albicans and *S. cerevisiae* were grown in YPD rich medium (2% peptone, 2% glucose, 1% Yeast Extract). SD minimal medium, was prepared by adding the appropriate amino acids to 0.67% Yeast Nitrogen Base without amino acids (Difco) and supplemented with 2% glucose or 3% glycerol + 2% ethanol as carbon sources. This medium contains 100 μg/l iron from YNB and ferrozine was added for iron depletion. Growth of *C. albicans* in iron-poor media was carried out in modified Lee defined medium (Elorza *et al.*, 1988) supplemented with uridine (25 μg/ml); all the components used for preparing this medium contain no more than 0.0003% of total Fe as contamination. Water used to prepare media was of MilliQ quality. For iron starvation of *C. albicans*, ferrozine in SD media or DPD in Lee media (Morrisey *et al.*, 1996) were used as iron chelators. *E. coli* was grown in LB medium (1% peptone, 0.5% Yeast Extract, 0.5% NaCl).

DNA manipulations

Restriction endonucleases and T4 DNA ligase were used according to the recommendations of the suppliers. DNA fragments were analysed on 0.6–1.2% horizontal agarose gels. Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (1979). DNA fragments were isolated from agarose gels by the freeze–squeeze method of Tautz and Renz (1983). Plasmids were isolated from yeast according to Rose *et al.* (1990). Probes for Southern analysis were labelled by the digoxygenin method of Boehringer-Mannheim, either with random primer extension or PCR amplification, following the instructions provided by the manufacturer. Southern blot analysis was carried out as described by Nieto *et al.* (1993) and Northern blots as described in Casas *et al.* (1997), except that transfer was carried out by capillarity and RNA crosslinks were made by incubation for 30 min at 120°C.

Cell transformation

E. coli transformations were carried out according to Inoue *et al.* (1990). Yeast cells were transformed using the lithium acetate method of Ito *et al.* (1983), with modifications described by Rose *et al.* (1990).

Selection of Δ*aft1* wild-type transformant clones

S. cerevisiae CML126 carrying Δ*aft1* mutation is unable to grow in iron-deficient media when glycerol/ethanol were used as carbon sources. In these conditions, CML128 (*AFT1*) strain is able to grow. We used SD-glycerol/ethanol medium supplemented with 0.4 μM ferrozine, to select for CML126 transformant clones that complement or suppress Δ*aft1* mutation. The required amino acids were added according to Rose *et al.* (1990).

DNA sequencing and homology analyses

*Hin*dII–*Hin*dII and *Sca*I–*Sca*I overlapping fragments, derived from plasmid pIRO1, were subcloned in pUC18 and sequenced in an automated DNA sequencer (Applied Biosystems, Model 370, Servei de Secuençiació de la Universitat de Valèncía) by the dideoxy chain termination method, using *Taq* DNA polymerase, primed with pUC18/19 reverse and forward primers. The sequence was completed in both chains with specific oligonucleotides: IRO1 (CAGTAGCATCATCCTCAG); IRO2 (GTGTTAGTGGCTTCT); IRO3 (CCCCGTGT CATTGCTAG); and IRO4 (CTAGCAATGA CACGGGG), and deposited at GenBank (Accession No. AJO13192).

Ferric reductase and iron determinations

Ferric reductase was assayed according to Morrisey *et al*. (1996) and the results were reported by cell dry weight (*C. albicans*) or by number of cells (*S. cerevisiae*). For determination of dry weight, cells from a 25 ml exponential phase culture were collected on Millipore nitrocellulose filters (0.22 μm pore diameter). Filters were dried before and after culture filtration until constant weight. For intracellular iron assays, cells were grown in Lee medium until stationary phase and then collected by centrifugation at $3000 \times g$ and washed twice with MilliQ water. The last pellet was mixed with glass beads and the cells were broken by vigorous shaking and then resuspended in 1 ml MilliQ water. After centrifugation for 10 min at $1200 \times g$, the supernatant was lyophilized, resuspended in 300 μl MilliQ water and used for iron quantification according to Fish (1988).

Siderophore utilization

Bacterial siderophore ferrichromes were assayed according to Ismail and Lupan (1986).

Flow cytometry

All the flow cytometric procedures were performed on an EPICS XL-MCL analyser (Beckman-Coulter, Hialeah, FL, USA) with a 15 mW air-cooled argon-ion laser tuned at 488 nm (Gil *et al*., 1996).

For determination of cell concentration, cell suspensions from cultures at the different experimental points were diluted 1/10 in distilled water and thoroughly vortexed immediately prior to flow cytometric analysis. Yeast populations were identified and selected by their properties of forward and side light scatter (Gil *et al*., 1996). For absolute count determination, data acquisition was stopped automatically at 20 μl sample volume, and the number of events recorded. Cell concentration (cells/ml) in the original suspension was then calculated by multiplying the number of events by a factor of 500. To ensure the accuracy of the determination, the volume stop-based absolute counting was calibrated against a suspension of fluorescent beads of known concentration (Flow-Count, Beckman-Coulter, Hialeah, FL, USA). For simultaneous estimation of mitochondrial membrane potential (MMP) (Juan *et al*., 1994) and intracellular superoxide generation (Vowells *et al*., 1995), cell suspensions from cultures at the different experimental points were diluted 1/10 in distilled water and incubated with ^{123}rhodamine (RH123; Molecular Probes, Eugene, OR, 50 ng/ml final concentration, for estimation of MMP) and hydroethidine (HE; Molecular Probes, Eugene, OR, 5 μg/ml final concentration, for quantitation of intracellular superoxide) for 15 min at 37°C. Samples were thoroughly vortexed immediately prior to flow cytometric analysis. Yeast populations were identified and selected by their properties of forward and side light scatter. RH123 and HE fluorescences were collected for 20.000 cells.

Results

Isolation of a gene that suppress Δ*aft1* phenotype

As described in Materials and methods, we have developed an in-plate selection protocol for clones that complement or suppress an *S. cerevisiae* Δ*aft1* mutation. Transformation of CML126 strain with a genomic *C. albicans* DNA library produced about 500 tryptophan-prototrophic transformants. Among these, we selected two clones that allow growth of the mutant strain in iron-depleted media. Both clones, derived from independent transformations, bear an identical plasmid named pIRO1 (Figure 1). Complementation (suppression) of the Δ*aft1* mutation of CML126 by pIRO1 was linked to tryptophan prototrophy, as has been demonstrated in plasmid curing experiments (not shown). Growth in liquid SD iron-depleted media with increasing concentrations of ferrozine demonstrated that pIRO1 suppresses the poor growth phenotype of Δ*aft1* mutants in these conditions (Figure 2).

Analysis of pIRO1

The restriction map of the pIRO1 showed one insert of about 4 kb (Figure 1). The preliminary sequence of the pIRO1 *Eco*RI–*Hin*dIII fragment showed high homology with the *C. albicans URA3* gene. The comparison of pIRO1 with pAL2, a plasmid previously isolated by del Castillo *et al*. (1993) that contains the *C. albicans URA3* locus, shows a high similarity in the restriction maps of both plasmids (Figure 1) and transformation of strain CML126 with pAL2 shows that this plasmid is also capable of suppressing the Δ*aft1* phenotype (Figure 2).

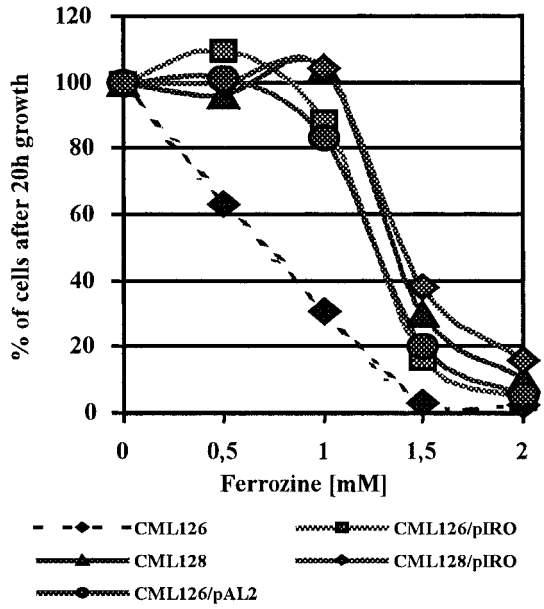

Figure 2. Growth in SD media containing increasing ferrozine concentrations. 100% corresponds to number of cells/ml in medium without ferrozine. Total cells were counted after 20 h growth

Despite suppression of Δ*aft1* phenotype we were not able to detect ferric reductase activity in CML126/pAL2, indicating that Iro1p does not activate gene transcription in the same way as Aft1p. However, we were able to detect ferric reductase activity in a strain bearing a deletion in *MAC1* (Georgatsou *et al.*, 1997), a gene implicated in regulation of iron and copper uptake (Table 1).

The DNA sequence of the pIRO1 *Eco*RI–*Sal*I region extends over 1826 bp. The sequence shows a unique ORF between positions 191 and 1729, which we designated *IRO1* (GenBank Accession No. AJO13192).

Genomic localization and expression of the gene *CaIRO1*

In order to locate the *CaIRO1* gene in the genome of *C. albicans*, Southern analyses were performed, using as probes a pIRO1 *Hin*cII–*Hin*cII fragment, corresponding to the *IRO1* ORF in the pIRO1 insert, and the *CaURA3* probe from pAL2 (del Castillo Agudo *et al.*, 1993). Hybridization was done with chromosomal DNA of the ATCC26555 strain digested with the enzymes *Eco*RI, *Hin*dIII or *Xba*I. The bands detected with both probes were coincident, with the exception of additional bands observed in *Eco*RI-digested DNA with *URA3* probe (Figure 3A). These differences are caused by an *Eco*RI restriction polymorphism in this region (Nieto and del Castillo, 1991). We can conclude that *CaIRO1* is strongly linked to the gene *URA3* of *C. albicans*. This result correlates with the presence of both genes in plasmids pAL2 and pIRO1 (Figure 1) and was confirmed by the overlapping of DNA sequences from *IRO1* and *URA3* in pIRO1 and of *IRO1* and published sequences of the *URA3* locus.

Northern blot experiments have shown that this gene is transcribed in *C. albicans* growing cells, and that its transcription is not regulated by the concentration of iron in the medium (Figure 3C).

Sequence analysis of the *CaIRO1* protein

The amino acid sequence deduced from the *CaIRO1* ORF is presented in Figure 4. The sequence does not show any significant homology with any other sequence available in the databases. However, the deduced protein sequence has some interesting features:

(i) The protein is very rich (22.62%) in serine and threonine. These amino acids are located mainly in the C-terminal region, where clusters of threonine and serine are present (Figure 4).
(ii) Glutamate and aspartate residues account for 19.5% of the total amino acids and are located mainly at the N-terminal region (Figure 4). Consequently, this protein is acidic (pH 4.02), hydrophilic and polar.

Table 1. Ferric reductase activity in Δaft1, Δ*mac1* and the corresponding pAL2 transformant strains

Strains						
AFT1 wild-type (CML128)	Δ*aft1*	Δ*aft1*/pAL2	*MAC1-FRE1-FRE2* wild-type	Δ*mac1*	Δ*mac1*/pAL2	*fre1/fre2*
21.4	5.47	7.41	50	6.12	46.73	7.96

Units are defined as nmol Fe(II)/min/10^6 cells. Mean values from three different assays.

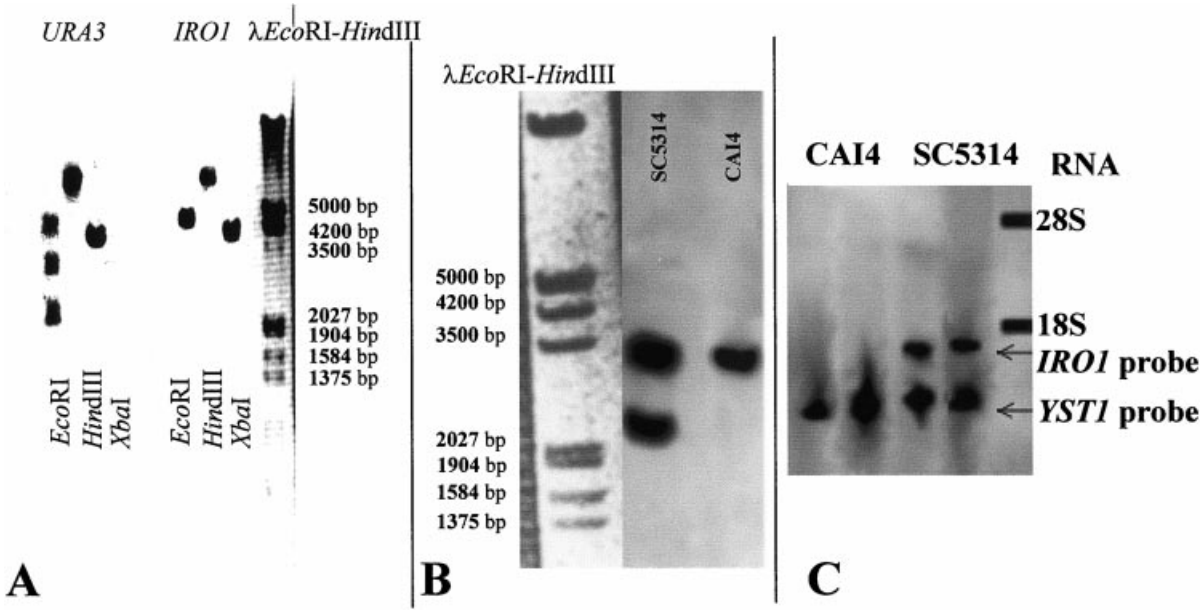

Figure 3. (A) Southern blot of genomic DNA from *C. albicans* ATCC26555, with URA3 and IRO1 probes. (B) Southern blot of DNA from strains SC5314 and CAI4 digested with *Eco*RV and hybridized with a *Hin*cII–*Hin*cII *IRO1* probe containing the *Eco*RV site. (C) Northern blot of total RNA from strains SC5314 and CAI4, probed sequentially with *IRO1* and *YST1* probes (Montero *et al.*, 1998), is a constitutively expressed *C. albicans* gene. YST1 probe was kindly provided by Dr Eulogio Valentin from our Department. Cell growth in media supplemented with 2 mM Fe^{2+} or in iron-depleted media

(iii) The protein shares a polyglutamic motif and several serine–threonine motifs that could be of relevance for the physiological function of the protein. Polyglutamate motifs are found in the products of of *S. cerevisiae* (Feaver *et al.*, 1994) and *C. albicans TFA1* (a subunit of the transcriptional factor *TFIIE* gene). Serine/threonine-rich motifs were also found in *C. albicans TFA1* (sequence obtained from the *C. albicans* DNA sequences database: **http://candida.stanford.edu/bin/gbrowse2/265092-265092B07**).

Physiological study in *C. albicans*

The *C. albicans* strain CAI4 (Fonzi and Irwin, 1993), carrying a deletion in the *URA3* gene, is usually employed in genetic studies, mainly for disruption of other genes in *C. albicans* (see e.g. Balan *et al.*, 1997; Fonzi and Irwin, 1993; Talibi and Raymond, 1999; Zhao *et al.*, 1996). As a consequence of the linkage between *URA3* and *IRO1*, the CAI4 Δ*ura3* strain has also lost the 3′ half of *IRO1* gene. According to Fonzi and Irwin (1993), the *URA3* deletion in CAI4 comprises the *Eco*RV–*Xba*I region of the *URA3–IRO1* loci (see Figure 1). In agreement with the published data, CAI4 DNA digested with *Eco*RV and probed with the *IRO1 Hin*cII–*Hin*cII fragment has lost a band, as expected if the deletion is flanked by the *Eco*RV site in *IRO1* ORF (Figure 3B). *IRO1* mRNA can not be detected by Northern analysis in CAI4 strain (Figure 3C).

It has also been reported that CAI4 has lost its virulence in animal models (Cole *et al.*, 1995). The strain has other particular phenotypes traits. On the one hand, CAI4 tends to form mycelia in iron-limited Lee medium without previous induction. This phenotype is partially suppressed when the medium is supplemented with 2 mM ferric citrate and 300 μM DPD (Figure 5). In the same conditions, SC5314 (the parental strain of CAI4) grows in the yeast form.

Growth differences between CAI4 and SC5314 were observed in SD medium supplemented with different carbon sources and in presence or absence of ferrozine. At low concentrations of the chelator (Table 2A), CAI4 shows reduced growth in glucose, a phenotype similar to *S. cerevisiae* Δ*aft1* strains. However, at high concentrations of ferrozine CAI4 does not seem to be more affected than the parental

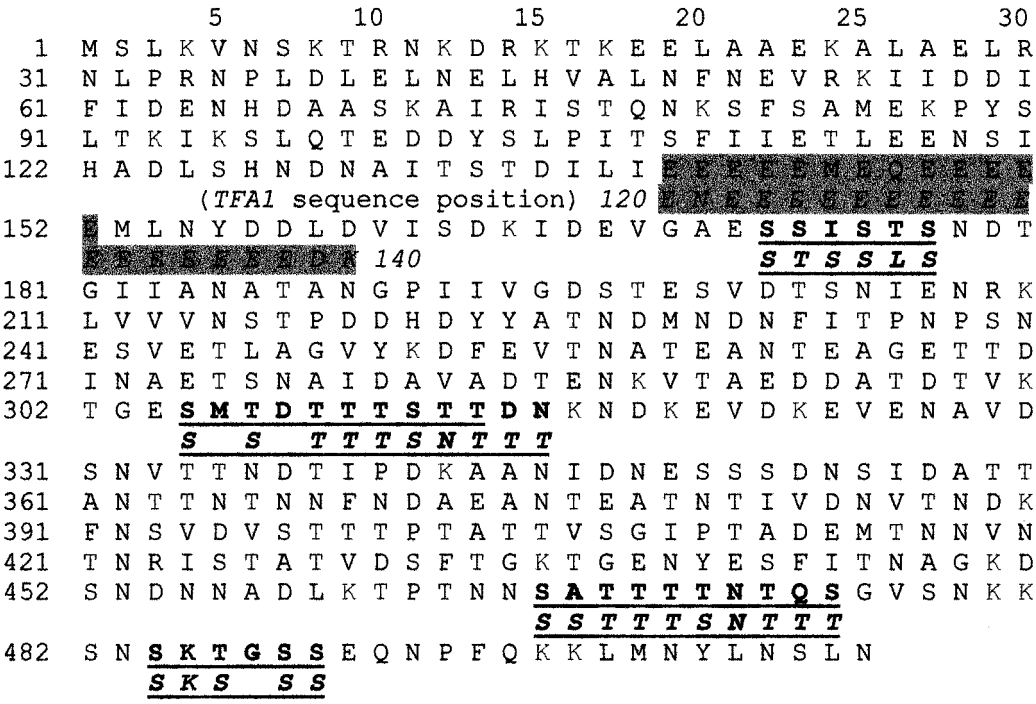

Figure 4. Amino acid sequence of Iro1p. The polyglutamic motif located between amino acids 140–151 in *IRO1* and 120–139 in *TFA1* are highlighted. The serine/threonine-rich motifs underlined do not correlate in position between the two genes. Sequence motifs from *TFA1* polyE motif and serine/threonine-rich motifs are represented in italics under the homologous regions in *IRO1*

strain SC5314 (Table 2A). The colour of the medium shifted to yellow when the wild-type strain, SC5314, was grown in glucose or galactose in the presence of ferrozine. This colour indicates the presence of a ferrozine–Fe^{3+} complex in the medium. CAI4 is unable to promote this colour change in the media (data not shown).

In respiratory growth conditions (glycerol/ethanol as carbon source), CAI4 hardly reaches 30% of the growth of the wild-type strain. Surprisingly, ferrozine concentrations higher than 1 mM seems to stimulate CAI4 growth, allowing better growth of the mutant strain, in these conditions, than the wild-type strain (Table 2A). Stimulation of CAI4 growth by ferrozine was also observed when galactose was used as carbon source (Table 2A). These results have been confirmed in Lee medium using DPD as a chelator and determining the cell concentration with a flow cytometer (Table 2B).

Despite the fact that CAI4 shows a relative growth stimulation by iron chelating agents, this strain is more sensitive to iron depletion than the parental strain, with a minimal inhibitory concentration (MIC) slightly lower than that of SC5314 (Table 2C).

CAI4 growth seems also be more stimulated by the presence of bacterial siderophore ferrichrome in extreme conditions of iron depletion; showing a higher ability to capture iron–ferrichrome complexes in concentrations of 125 and 150 μM DPD in glycerol/ethanol media (data not shown), while the MIC of DPD is 30 μM in these conditions (Table 2C).

Iron uptake in strain CAI4

In order to evaluate the effect of *IRO1* on the activity of enzymes involved in iron transport, we have determined ferric reductase activity, which is transcriptionally controlled by *AFT1* in *S. cerevisiae*. In conditions of iron depletion (Lee medium plus 30 μM DPD), SC5314 shows an increased activity of about 2.5-fold that of CAI4. However, no significant differences were observed in internal iron contents, either in iron-rich or iron-depleted media (data not shown).

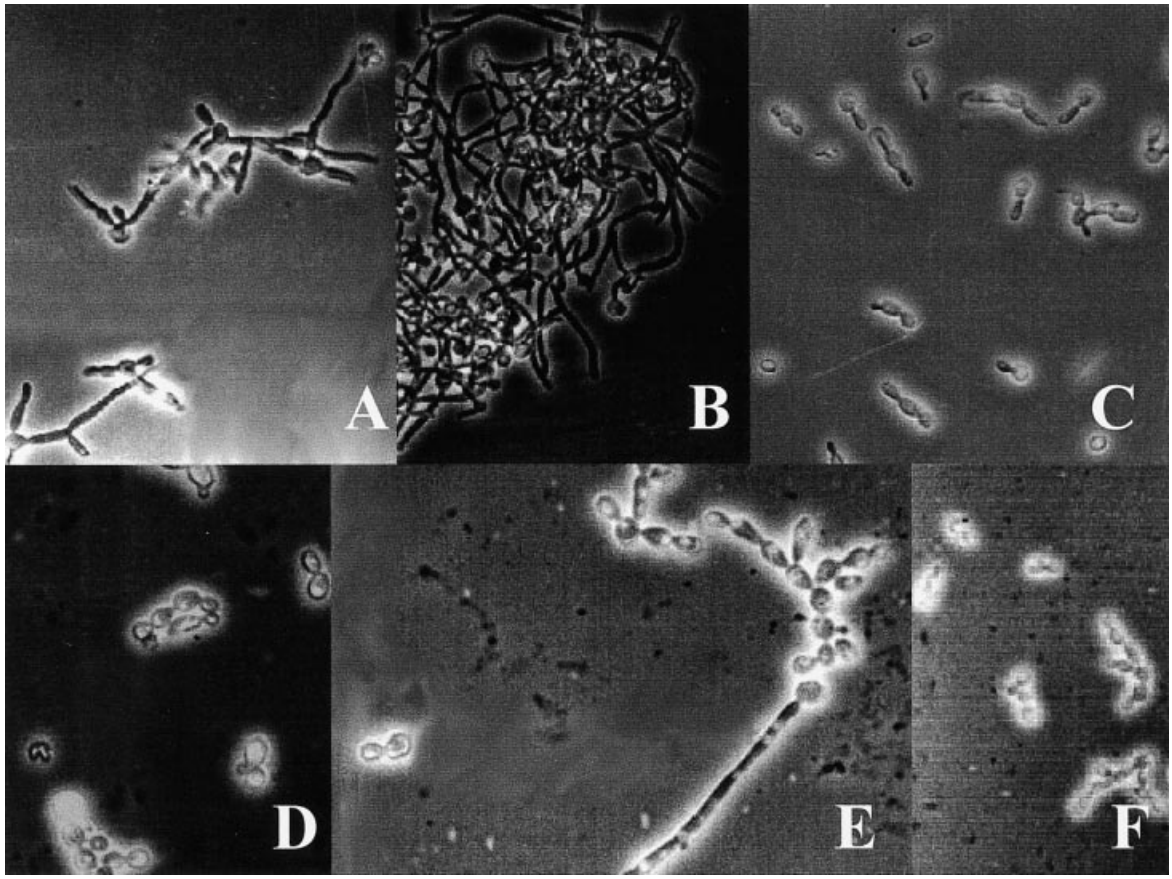

Figure 5. Mycelial growth of *C. albicans* CAI4 in Lee medium (A, B). Growth in medium supplemented with 2 mM Fe^{2+} (D, E). Growth of the parental strain SC5314 in the same conditions, (C) in Lee medium and (F) in Lee medium + 2 mM Fe^{2+}, respectively

Table 2. Maximal growth of *C. albicans* strains SC5314 and CAI4 in different conditions of iron content and carbon source

	Glucose		**Glycerol/ethanol**		**Galactose**	
	SC5314	**CAI4**	**SC5314**	**CAI4**	**SC5314**	**CAI4**
A: Growth (OD) in SD with different carbon sources and ferrozine concentration						
Ferrozine (mM)						
0	11.6	9.9	9.8	2.9	9.8	3.9
0.4	4.9	2.1	4.2	2.9	4.2	2.9
1	3.4	3.8	2.3	4.6	2.3	4.6
2	3.4	3.7	2	4.2	2	4.2
B: Growth (cells/ml × 10^6) in Lee medium and different carbon sources and iron amounts						
ϕ	1951	772	631	630	ND	ND
300 μM Fe^{2+}	2078	598	371	716,8	ND	ND
30 μM DPD	1856	833	728	1065	ND	ND
C: Sensibility to iron depletion as DPD MIC						
MIC of DPD (μM)	90	70	70	30	ND	ND

Growth was quantified at stationary phase as optical density measurement (A), or by cell counting using a flow cytometer (B). Minimal inhibitory concentration (MIC) of DPD growth is expressed in μM (C). ND, not determined.

We also evaluated the mitochondrial membrane potential and intracellular generation of superoxide in the two strains, either in fermentative or respiratory growth conditions, in iron-depleted media or supplemented with 300 μM Fe^{2+} (as ferrous citrate). No significant differences were observed between the two strains in equivalent media (data not shown).

Discussion

We have isolated a *C. albicans* gene *IRO1* by complementation (suppression) of the Δ*aft1* mutation in *S. cerevisiae*. The bulk of the results presented, although pointing to differences in iron metabolism between CAI4 and the parental strain SC5314, are not sufficient to assign any specific function to *IRO1*. The lack of sequence homology between *IRO1* and *S. cerevisiae AFT1* gene, indicates that re-establishment of the wild phenotype of the CML126 mutant (growth in iron-deficient medium), is a consequence of suppression (and not complementation) of the mutation. Therefore, it is probable that *IRO1* may play a different role in *C. albicans* to that of *AFT1* in *S. cerevisiae*.

Nevertheless, the characteristics of the deduced sequence of the protein indicates a possible role as a transcription factor. The relationship to iron uptake is also unclear, since CAI4 shows better behaviour than the wild-type SC5314 in extreme iron-deficient conditions. Thus, a role of Iro1p in the control of a highly efficient mechanism of iron uptake in *C. albicans*, might be proposed, independent or complementary to the ferric-reductase-dependent iron transport system. This mechanism would be operating only in very extreme conditions of iron depletion. We can hypothesize, on the basis of relative growth stimulation by chelating agents (DPD and ferrichrome), that this mechanism of iron transport would be specific for chelated iron, i.e. the form in which this element is present in the host. It could also explain the high tolerance of *C. albicans* to ferrozine (MIC > 2 mM) as compared to *S. cerevisiae* (MIC < 0.2 mM).

According to the characteristics of Iro1p (presence of potential phosphorylation sites, acidic pH, presence of polyglutamic- and serine/threonine-rich clusters), this protein could play a role as a transcription factor, which would modulate the expression of the putative iron transport mechanism associated with extreme conditions of iron depletion, thus allowing their expression only in very severe iron deficiency. This mechanism could play a role in *C. albicans* virulence, as lack of *IRO1* function seems to induce mycelium formation in iron-depleted medium, although this might be the consequence of a general response to stress, and not a specific response to iron deficiency.

Beyond the interest of *IRO1* as a gene potentially implicated in iron uptake, and maybe in other stress responses, considerations must be given to the loss of virulence of CAI4, and other *ura3* mutants of *C. albicans*. Previous work attributed the loss of virulence of *ura3*-deficient strains to uridine auxotrophy (Cole *et al.*, 1995; Kirsch and Whitney, 1991). However, although different backgrounds were used in the two studies, all *ura3* mutants were obtained by gene disruption. Kirsh and Whitney (1991) reported that the loss of virulence observed in the *ura3* strain studied by them was not completely recovered when a *URA3* gene was reintroduced in the mutant strain. These authors suggested that putative, additional mutations, generated by gene disruption, may be implicated in virulence loss by the strain they used (Kirsch and Whitney, 1991). In the case of CAI4, although there are no specific studies, results published by different authors suggest that reinsertion of *URA3* in CAI4 never restored virulence completely (see e.g. Lay *et al.*, 1998; Wysong *et al.*, 1998). In our laboratory, work is in progress to construct defined mutations at the *URA3* locus in order to determine the role of this gene and *IRO1* in *C. albicans* pathogenicity.

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